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(54) Title: FUNCTIONALIZED NANOCRYSTALS AND THEIR USE IN DETECTION SYSTEMS

(57) Abstract

Provided are compositions comprising water–soluble, functionalized nanocrystals. The water–soluble functionalized nanocrystals comprise quantum dots capped with a layer of a capping compound, and further comprise, by operably linking and in a successive manner, one or more additional compounds. Preferably, an additional compound comprises diaminocarboxylic acid which is operatively linked to the capping compound, and may further comprise an amino acid, and affinity ligand, or a combination thereof. Also provided are methods of using the functionalized nanocrystals having an affinity ligand to detect the presence or absence of a target substrate in a sample by contacting the functionalized nanocrystals with the sample so that complexes are formed between the functionalized nanocrystals and substrate, if the substrate is present; exposing the complexes in the detection system to an excitation light source, and detecting the emitted fluorescence peak.

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FUNCTIONALIZED NANOCRYSTALS AND THEIR USE IN DETECTION SYSTEMS

FIELD OF INVENTION

This invention relates to novel compositions comprising functionalized nanocrystals. More particularly, the present invention relates to water-soluble nanocrystals which have a coat comprising a capping compound, and one or more additional compounds successively overlayered onto the capped nanocrystal. The present invention also relates to the use of the functionalized nanocrystals for providing a detectable signal in detection systems in which the nanocrystals are employed.

BACKGROUND OF THE INVENTION

Nonisotopic detection systems have become a preferred mode in scientific research and clinical diagnostics for the detection of biomolecules using various assays including flow cytometry, nucleic acid hybridization, DNA sequencing, nucleic acid amplification, immunoassays, histochemistry, and functional assays involving living cells. In particular, while fluorescent organic molecules such as fluoroscein and phycoerythrin are used frequently in detection systems, there are disadvantages in using these molecules in combination. For example, each type of fluorescent molecule typically requires excitation with photons of a different wavelength as compared to that required for another type of fluorescent molecule. However, even when a single light source is used to provide a single excitation wavelength (in view of the spectral line width), often there is insufficient spectral spacing between the emission optima of different fluorescent molecules to permit individual and

quantitative detection without substantial spectral overlap. Further, currently available nonisotopic detection systems typically are limited in sensitivity due to the finite number of nonisotopic molecules which can be used to label a biomolecule to be detected.

Semiconductor nanocrystals ("quantum dots") are known in the art. Generally, quantum dots can be prepared which result in relative monodispersity (e.g., the diameter of the core varying approximately less than 10% between quantum dots in the preparation), as has been described previously. Examples of quantum dots are known in the art to have a core selected from the group consisting of CdSe, CdS, and CdTe (collectively referred to as "CdX").

CdX quantum dots have been passivated with an inorganic coating ("shell") uniformly deposited thereon. Passivating the surface of the core quantum dot can result in an increase in the quantum yield of the fluorescence emission, depending on the nature of the inorganic coating. The shell which is used to passivate the quantum dot is preferably comprised of YZ wherein Y is Cd or Zn, and Z is S, or Se. Quantum dots having a CdX core and a YZ shell have been described in the art. However, the above described quantum dots, passivated using an inorganic shell, have only been soluble in organic, non-polar (or weakly polar) solvents.

To make quantum dots useful in biological applications, it is desirable that the quantum dots are water-soluble. "Water-soluble" is used herein to mean sufficiently soluble or suspendable in a aqueous-based solution, such as in water or water-based solutions or buffer solutions, including those used in biological or molecular

detection systems as known by those skilled in the art. Typically, CdX core/YZ shell quantum dots are over-coated with trialkylphosphine oxide, with the alkyl groups most commonly used being butyl and octyl. One method to make the CdX core/YZ shell quantum dots water-soluble is to exchange this overcoating layer with a coating which will make the quantum dots water-soluble. For example, a mercaptocarboxylic acid may be used to exchange with the trialkylphosphine oxide coat. Exchange of the coating group is accomplished by treating the water-insoluble quantum dots with a large excess of neat mercaptocarboxylic acid. Alternatively, exchange of the coating group is accomplished by treating the water-insoluble quantum dots with a large excess of mercaptocarboxylic acid in $CHCl_3$ solution. The thiol group of the new coating molecule forms Cd (or Zn)-S bonds, creating a coating which is not easily displaced in solution. Another method to make the CdX core/YZ shell quantum dots water-soluble is by the formation of a coating of silica around the dots. An extensively polymerized polysilane shell imparts water solubility to nanocrystalline materials, as well as allowing further chemical modifications of the silica surface. However, depending on the nature of the coating group, quantum dots which have been reported as water-soluble may have limited stability in an aqueous solution, particularly when exposed to air (oxygen) and/or light. More particularly, oxygen and light can cause the molecules comprising the coating to become oxidized, thereby forming disulfides which destabilize the attachment of the coating molecules to the shell. Thus, oxidation may cause the coating molecules to migrate away from the surface of the nanocrystals, thereby exposing the surface of the

nanocrystals in resulting in "destabilized nanocrystals". Destabilized nanocrystals form aggregates when they interact together, and the formation of such aggregates eventually leads to irreversible flocculation of the nanocrystals (e.g., see FIG. 1A).

Thus, there remains a need for a semiconductor nanocrystal which (a) is water-soluble; (b) is functionalized to enhance stability in aqueous solutions; (c) is a class of semiconductor nanocrystals that may be excited with a single wavelength of light resulting in detectable luminescence emissions of high quantum yield and with discrete luminescence peaks; and (d) is functionalized so as to be both water-soluble, and able to bind ligands, molecules, or probes of various types for use in an aqueous-based environment.

SUMMARY OF THE INVENTION

The present invention provides a composition comprising functionalized nanocrystals for use in non-isotopic detection systems. The composition comprises quantum dots (capped with a layer of a capping compound) that are water-soluble and functionalized by operably linking, in a successive manner, one or more additional compounds. In a preferred embodiment, the one or more additional compounds form successive layers over the nanocrystal. More particularly, the functionalized nanocrystals comprise quantum dots capped with the capping compound, and comprise a coating (a plurality of molecules comprising) diaminocarboxylic acid which is operatively linked to the capping compound. Thus, the functionalized nanocrystals may have a first layer comprising the capping compound, and a

second layer comprising diaminocarboxylic acid; and may further comprise one or more successive layers including a layer of amino acid, a layer of affinity ligand, or multiple layers comprising a combination thereof. The composition comprises a class of quantum dots that can be excited with a single wavelength of light resulting in a detectable luminescence emissions of high quantum yield and with discrete luminescence peaks.

In a method of detection of a target substrate using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals are further functionalized by binding an affinity ligand thereto. The resultant functionalized nanocrystals are placed in contact with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity. Contact, and subsequent binding, between the affinity ligand of the functionalized nanocrystals and the substrate, if present in the sample, results in a complex comprising the functionalized nanocrystal-substrate which can emit a detectable signal for quantitation, visualization, or other form of detection.

The above and other objects, features, and advantages of the present invention will be apparent in the following Detailed Description of the Invention when read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** is a bar graph comparing the stability of capped quantum dots ("W-SN") to the stability of functionalized nanocrystals ("FN") under oxidizing conditions.

FIG. **1B** is a bar graph comparing the non-specific binding of capped quantum dots ("W-SN") to the non-specific binding of functionalized nanocrystals ("FN").

FIG. **2** is a schematic illustrating chemically modifying a water-soluble quantum containing a layer of a capping compound to further comprise a layer of a diaminocarboxylic acid, and a layer of an affinity ligand (e.g., avidin).

FIG. **3** is a schematic illustrating chemically modifying a water soluble quantum dot containing a layer of a capping compound to further comprise a layer of a diaminocarboxylic acid, an additional layer of a diaminocarboxylic acid, and a layer of an affinity ligand.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

By the term "substrate" is meant, for the purposes of the specification and claims to refer to a molecule of an organic or inorganic nature, the presence and/or quantity of which is being tested for; and which contains a molecular component (domain or sequence or epitope or portion or chemical group or determinant) for which the affinity ligand has binding specificity. The molecule may include, but is not limited to, a nucleic acid molecule, protein, glyco-protein, eukaryotic or prokaryotic cell, lipoprotein, peptide, carbohydrate, lipid, phospholipid, aminoglycans, chemical messenger, biological receptor, structural component, metabolic product, enzyme, antigen, drug, therapeutic, toxin, inorganic chemical, organic chemical, and the like. The substrate may be *in vivo*, *in vitro*, *in*

situ, or *ex vivo*. A preferred substrate may be used to the exclusion of a substrate other than the preferred substrate.

By the term "affinity ligand" is meant, for purposes of the specification and claims, to mean a molecule which has binding specificity and avidity for a molecular component of, or associated with, a substrate. In general, affinity ligands are known to those skilled in the art to include, but are not limited to, lectins or fragments (or derivatives) thereof which retain binding function; monoclonal antibodies ("mAb", including chimeric or genetically modified monoclonal antibodies (e.g., "humanized")); peptides; aptamers; nucleic acid molecules (including, but not limited to, single stranded RNA or single-stranded DNA, or single-stranded nucleic acid hybrids); avidin, or streptavidin, or avidin derivatives; and the like. The invention may be practiced using a preferred affinity ligand (e.g., a lectin) to the exclusion of affinity ligands other than the preferred affinity ligand. The term "monoclonal antibody" is also used herein, for purposes of the specification and claims, to include immunoreactive fragments or derivatives derived from a mAb molecule, which fragments or derivatives retain all or a portion of the binding function of the whole mAb molecule. Such immunoreactive fragments or derivatives are known to those skilled in the art to include $F(ab')_2$, Fab', Fab, Fv, scFV, Fd' and Fd fragments. Methods for producing the various fragments or derivatives from mAbs are well known in the art. For example, $F(ab')_2$ can be produced by pepsin digestion of the monoclonal antibody, and Fab' may be produced by reducing the disulfide bridges of $F(ab')_2$ fragments. Fab fragments can be produced by papain digestion of the monoclonal antibody, whereas Fv can be prepared

according to methods described in U.S. Patent No. 4,642,334. Single chain antibodies can be produced as described in U.S. Patent No. 4,946,778. The construction of chimeric antibodies is now a straightforward procedure in which the chimeric antibody is made by joining the murine variable region to a human constant region. Additionally, "humanized" antibodies may be made by joining the hypervariable regions of the murine monoclonal antibody to a constant region and portions of variable region (light chain and heavy chain) sequences of human immunoglobulins using one of several techniques known in the art. Methods for making a chimeric non-human/human mAb in general are known in the art (see, e.g., U.S. Patent No. 5,736,137). Aptamers can be made using methods described in U.S. Patent No. 5,789,157. Lectins, and fragments thereof, are commercially available. Lectins are known to those skilled in the art to include, but are not limited to, one or more of Aleuria aurantia lectin, Amaranthus caudatus lectin, Concanavalin A, Datura stramonium lectin, Dolichos biflorus agglutinin, soybean agglutinin, Erythrina cristagalli lectin, Galanthus nivalis lectin, Griffonia simplicifolia lectins, Jacalin, Macckia amurensis lectins, Maclura pomifera agglutinin, Phaeolepiota aurea lectins 1 and 2, Phaseolus vulgaris lectins, Ricin A, Moluccella laevis lectin, peanut agglutinin, Bauhinia purpurea agglutinin, Ricinus communis agglutinins, Sambucus nigra lectin, Vicia villosa agglutinin, Sophora japonica agglutinin, Caragana arborescens agglutinin, Helix aspersa agglutinin, Limax flavus lectin, limulin, wheat germ agglutinin, and Ulex europaeus agglutinin. A preferred affinity ligand may be used to the exclusion of an affinity ligand other than the preferred affinity ligand.

By the term "operably linked" is meant, for purposes of the specification and claims to refer to fusion or bond or an association of sufficient stability to withstand conditions encountered in a method of detection, between a combination of different molecules such as, but not limited to, between the quantum dot and a capping compound, between a capping compound and a diaminocarboxylic acid, between a diaminocarboxylic acid and a diaminocarboxylic acid, between a diaminocarboxylic acid and an affinity ligand, between a diaminocarboxylic acid and an amino acid, and between an amino acid and an affinity ligand, and a combination thereof. As known to those skilled in the art, and as will be more apparent by the following embodiments, there are several methods and compositions in which two or more molecules may be operably linked utilizing reactive functionalities. Reactive functionalities include, but are not limited to, bifunctional reagents/linker molecules, biotin, avidin, free chemical groups (e.g., thiol, or carboxyl, hydroxyl, amino, amine, sulfo, etc.), and reactive chemical groups (reactive with free chemical groups). A preferred reactive functionality may be used to the exclusion of a reactive functionality other than the preferred reactive functionality.

By the term "linker" is meant, for purposes of the specification and claims to refer to a compound or moiety that acts as a molecular bridge to operably link two different molecules, wherein one portion of the linker is operably linked to a first molecule, and wherein another portion of the linker is operably linked to a second molecule. The two different molecules may be linked to the linker in a step-wise manner. There is no particular size or

content limitations for the linker so long as it can fulfill its purpose as a molecular bridge. Linkers are known to those skilled in the art to include, but are not limited to, chemical chains, chemical compounds, carbohydrate chains, peptides, haptens, and the like. The linkers may include, but are not limited to, homobifunctional linkers and heterobifunctional linkers. Heterobifunctional linkers, well known to those skilled in the art, contain one end having a first reactive functionality to specifically link a first molecule, and an opposite end having a second reactive functionality to specifically link to a second molecule. As illustrative examples, to operably link a hydroxyl group of a polynucleotide strand to an amino group of a diaminocarboxylic acid, the linker may have: a carboxyl group to form a bond with the polynucleotide, and a carboxyl group to form a bond with the diaminocarboxylic acid. Heterobifunctional photo-reactive linkers (e.g., phenylazides containing a cleavable disulfide bond) are known in the art. For example, a sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate contains a N-hydroxy-succinimidyl group reactive with primary amino groups, and the phenyl-azide (upon photolysis) reacts with any amino acids. The linker may further comprise a protective group which blocks reactivity with a functional group on the linker which is used to react with and bind to a molecule to be linked. A deprotection reaction may involve contacting the linker to one or more conditions and/or reagents which removes the protective group, thereby exposing the functional group to interact with the molecule to be linked. Depending on the nature of the protective group, deprotection can be achieved by various methods known in the art, including, but not

limited to photolysis, acidolysis, hydrolysis, and the like. Depending on such factors as the molecules to be linked, and the conditions in which the method of detection is performed, the linker may vary in length and composition for optimizing such properties as flexibility, stability, and resistance to certain chemical and/or temperature parameters. For example, short linkers of sufficient flexibility include, but are not limited to, linkers having from 2 to 10 carbon atoms. A preferred linker may be used to the exclusion of a linker other than the preferred linker.

By the term "diaminocarboxylic acid" is meant, for purposes of the specification and claims to refer to an amino acid that has two free amine groups. The amino acid may be a naturally occurring amino acid, a synthetic amino acid, a modified amino acid, an amino acid derivative, and an amino acid precursor (e.g., citrulline and ornithine are intermediates in the synthesis of arginine). In a preferred embodiment, the diaminocarboxylic acid contains neutral (uncharged) polar functional groups which can hydrogen bond with water, thereby making the diaminocarboxylic acid (and the quantum dot to which it is made a part of) relatively more soluble in aqueous solutions containing water than those with nonpolar functional groups. Exemplary diaminocarboxylic acids include, but are not limited to, lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, and synthetic diaminocarboxylic acids such as 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid. A preferred diaminocarboxylic acid may be used to the exclu-

sion of a diaminocarboxylic acid other than the preferred diaminocarboxylic acid.

By the term "amino acid" is meant, for purposes of the specification and claims to refer to a molecule that has at least one free amine group and at least one free carboxyl group. The amino acid may have more than one free amine group, or more than one free carboxyl group, or may further comprise one or more free chemical reactive groups other than an amine or a carboxyl group (e.g., a hydroxyl, a sulfhydryl, etc.). The amino acid may be a naturally occurring amino acid, a synthetic amino acid, a modified amino acid, an amino acid derivative, and an amino acid precursor. The amino acid may further be selected from the group consisting of a monoaminocarboxylic acid, and a diaminocarboxylic acid. In a preferred embodiment, the monoaminocarboxylic acid contains one or more neutral (uncharged) polar functional groups which can hydrogen bond with water, thereby making the monoaminocarboxylic acid (and the quantum dot to which it is made a part of) relatively more soluble in aqueous solutions containing water than those with non-polar functional groups. Exemplary mono-aminocarboxylic acids include, but are not limited to, glycine, serine, threonine, cysteine, β-alanine, homoserine, and γ-aminobutyric acid. A preferred amino acid may be used to the exclusion of an amino acid other than the preferred amino acid.

By the term "capping compound" is meant, for purposes of the specification and claims to refer to a compound having the formula $HS(CH_2)_nX$, wherein X is a carboxylate (carboxylic moiety); or the formula $HS(CH_2)_nYX$, wherein X is a carboxylate and Y is an amine; as will be more apparent

from the following descriptions. "n" is a number in the range of from 1 to about 20, and preferably greater than 4. The thiol group of the capping compound forms Cd (or Zn)-S bonds (depending on whether the shell is Cd or Zn), creating a layer which is not easily displaced in solution. Additionally, the carboxylic acid moiety of the capping compound imparts some water solubility to the quantum dots. Exemplary capping compounds according to the present invention include, but are not limited to, mercaptocarboxylic acid, or mercaptofunctionalized amines (e.g., aminoethanethiol-HCl, homocysteine, or 1-amino-2-methyl-2-propanethiol-HCl). A preferred capping compound may be used to the exclusion of a capping compound other than the preferred capping compound.

The present invention provides compositions which can be used to generate a detectable signal comprising a light emission (e.g., fluorescence emission) of high quantum yield, thereby considerably improving the sensitivity of a non-isotopic detection system. According to the present invention, functionalized nanocrystals comprise quantum dots (core and shell) which comprises a first additional layer or coating comprising a capping compound, and a second layer or coating comprising diaminocarboxylic acid. In another embodiment of the present invention, functionalized nanocrystals comprise quantum dots which comprise a first layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and an addition comprising affinity ligand (one or more molecules of affinity ligand). In another embodiment of the present invention, functionalized nanocrystals comprise quantum dots which comprising a first

layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and a third layer comprising amino acid. In yet another embodiment of the present invention, functionalized nanocrystals comprise quantum dots (core and shell) which comprise a first layer or coating comprising the capping compound, a second layer comprising diaminocarboxylic acid, a third layer comprising amino acid, and wherein the third layer has operably linked thereto one or more molecules of affinity ligand. In each of the embodiments, the component of each successive layer is operably linked to the component of any contacting layer, as will be more apparent from the figures and following description.

In one embodiment of a method for using the functionalized nanocrystals according to the present invention, the functionalized nanocrystal comprises quantum dots, the capping compound, diaminocarboxylic acid, and operably linked to diaminocarboxylic acid is one or more molecules of affinity ligand. The functionalized nanocrystals are first contacted with a sample under conditions suitable for the nanocrystals to contact and bind, via the affinity ligand portion, the substrate, if present, in the sample being analyzed for the presence or absence of the substrate. Alternatively, the functionalized nanocrystals may comprise quantum dots, the capping compound, diaminocarboxylic acid, amino acid, and affinity ligand operably lnked to the amino acid.

In another embodiment of a method for using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals comprise quantum dots, a coating of capping compound, and a coating comprising

diaminocarboxylic acid. The user may then operably link the desired affinity ligand to the diaminocarboxylic acid of the functionalized nanocrystal using methods known in the art. Alternatively, the functionalized nanocrystals may comprise quantum dots, a coating comprising the capping compound, a coating comprising diaminocarboxylic acid, and a coating comprising an amino acid; and the user may then operably link the desired affinity ligand to the amino acid of the functionalized nanocrystal using methods known in the art.

EXAMPLE 1

In one preferred embodiment, the composition according to the present invention comprises quantum dots which are capped by the addition of a layer comprising a capping compound, and more preferably a capping compound having the formula $HS(CH_2)_nX$, (wherein X is a carboxylic moiety), and comproises one or more successive layers comprising diaminocarboxylic acid, amino acid, or a combination thereof. Desirable features of the functionalized nanocrystals according to the present invention are that (a) can be excited with a single excitation light source, (b) when excited, emit a detectable light emission (e.g., fluorescence emission) of high quantum yield (e.g., a single quantum dot having at a fluorescence intensity at least a log greater than that of conventional fluorescent dye molecules), (c) have a light emission having a discrete fluorescence peak, and (d) are water-soluble. The functionalized nanocrystals typically should comprise a quantum dot particle of substantially uniform size of less than 100 Angstroms, and preferably have a substantially uniform size in the range of sizes of from about 2 nm to

about 10 nm (diameter). Preferred quantum dots used in the production of functionalized nanocrystals are comprised of a core of CdSe passivated with ZnS.

In this embodiment is illustrated the production of the functionalized nanocrystals. Exemplary quantum dots comprise a CdSe core, and a ZnS shell, "(CdSe)ZnS". TOPO capped CdSe were produced by placing TOPO (5g) in a vessel, and dried at 150°C for 1 hour under vacuum. The vessel was then backfilled with argon and heated to 300°C. In a controlled environment, $CdMe_2$ (7.2 µl , 0.1 mmol) and 1 M trioctylphosphine-Se solution (90 µl , 0.09 mmol) and trioctylphosphine (5 ml) were mixed, and then placed into an injector. This mixture was added to the TOPO in a reaction vessel, previously removed from the heat, in a single continuous injection with vigorous stirring, thereby resulting in the temperature decreasing to about 180°C. The reaction vessel was then subjected to heat to raise the temperature 5°C every 10 minutes. Aliquots may be removed from the reaction vessel at various time intervals (5 to 10 minutes) to monitor the increase in size of nanocrystals over time, by the observation of the absorption spectra. The temperature may be changed, or the reaction halted, upon reaching nanocrystals of the desired characteristics. For example, the reaction vessel was cooled to about 60°C, 40 ml of methanol was added to cause the nanocrystals to flocculate. After centrifugation, a brightly colored liquid layer of nanocrystals dissolved in trioctylphosphine remained. The methanol/TOPO layer was decanted off, and pyridine (10 ml) was added to the nanocrystal solution and allowed to stand for at least one hour. The nanocrystals were then precipitated as a powder by addition of hexanes, and separated

by centrifugation. The powder was washed once more with hexanes, then dissolved in 30 ml pyridine, and centrifuged to remove any reaction byproducts.

To prepare (CdSe)ZnS nanocrystals, the pyridine solution (30 ml) was placed in a reaction vessel, rigorously degassed with an inert gas (e.g., argon), and refluxed for one hour before adjusting the temperature to approximately 100°C. Equimolar amounts of diethyl zinc (zinc source) and hexamethyldisilathiane (sulfide source) were dissolved in trioctylphosphine (2-4 ml) in a controlled environment (glove box) and loaded into an injector. A reaction vessel containing the CdSe dots dispersed in pyridine was heated under an atmosphere of argon, and the Zn and S were added dropwise, via the injector, with vigorous stirring of the mixture for 5-10 minutes. The mixture was left stirring for several hours. After cooling, the pyridine solution was centrifuged to remove any insoluble material. The over-coated nanocrystals were stored in this solution to ensure that the surface of the nanocrystals remained passivated with pyridine.

To prepare nanocrystals which are capped, the pyridine overcoating of the (CdX) core/YZ shell nanocrystals were exchanged with a capping compound which contributes to the water-solubility of the resultant nanocrystals. For example, a capping compound comprising mercaptocarboxylic acid may be used to exchange with the pyridine overcoat. Exchange of the coating group is accomplished by treating the water-insoluble, pyridine-capped quantum dots with a large excess of neat mercapto-carboxylic acid. To accomplish this, the pyridine-capped (CdSe)ZnS quantum dots were precipitated with hexanes, and then isolated by centri-

fugation. The residue was dissolved in neat mercaptoacetic acid, with a few drops of pyridine added, if necessary, to form a transparent solution. The solution is allowed to stand at room temperature for at least six hours. Longer incubation times lead to increased substitution by the thiol. Overnight incubations are ideal. Chloroform is added to precipitate the nanocrystals and wash away excess thiol. The nanocrystals were isolated by centrifugation, washed once more with chloroform, and then washed with hexanes. The residue was briefly dried with a stream of argon. The resultant nanocrystals, coated with the capping compound, showed some solubility in water or other aqueous solutions. The nanocrystals, in an aqueous solution, were centrifuged once more, filtered through a 0.2 μm filter, degassed with argon, and stored in an amber vial. Failure to protect the nanocrystals, in solution, from air and light leads to rapid, irreversible flocculation.

Thus, single-site attachment of the capping compound (a mercaptocarboxylic acid; e.g., mercaptoacetic acid, mercaptopropionic acid, mercaptoundecanoic acid, etc.) suffers from limited stability in aqueous solution in the presence of water when exposed to air (oxygen) and light. It was found that by functionalizing the nanocrystal by adding a coating of diaminocarboxylic acid, resulted in significant enhancement of solubility and stability of the resultant functionalized nanocrystal. In that regard, as shown in FIG. 1A, the functionalized nanocrystals comprising a coat of diaminocarboxylic acid ("FN") unexpectedly show a significant increase in stability in an aqueous environment compared to quantum dots having an outer layer of just the

capping compound ("W-SN), when exposed over time to identical conditions of an oxidizing environment (e.g., light and air). Additionally, as shown in FIG. 1B, functionalized nanocrystals containing a coat of diaminocarboxylic acid ("FN") unexpectedly result in a significant decrease in non-specific binding compared to quantum dots having an outer layer of just the capping compound ("W-SN), when each were contacted with a surface that is both hydrophilic and hydrophobic (e.g., as may be encountered in a detection system), followed by washing of the surface, followed by detection of residual nanocrystals (as measured by number of events of fluorescence versus the intensity of fluorescence; using a fluorescence microscope with a video camera attachment, time of exposure- $1/30^{th}$ of a second).

Thus, in a preferred embodiment, the diaminocarboxylic acid (a) enhances the water-solubility of the functionalized nanocrystal; (b) has at least two free functional groups which are carboxyl-reactive, thereby enabling the diaminocarboxylic acid molecule to operably link to and crosslink carboxyl groups extending from the capping compound on the capped quantum dots; and (c) once operably linked to the capping compound, has one or more free functional groups which can be used for operably linking affinity ligand thereto. Additionally, a free carboxylic acid group on the diaminocarboxylic acid will remain as a site for attachment (operably linking) of other molecules to the diaminocarboxylic acid layer. In a more preferred embodiment, the diaminocarboxylic acid comprises lysine (2,6-diaminohexanoic acid).

For operably linking diaminocarboxylic acid to the capping compound of capped quantum dots, commercially avail-

able crosslinking agents and methods known to those skilled in the art may be used. For example, and as illustrated in FIG. 2, mercaptoacetic acid-capped nanocrystals were dissolved in an aqueous buffer system (pH of about 7). The buffer may comprise such buffers as PBS or HEPES; however, the presence of phosphate may dramatically decrease the lifetime of the crosslinking agent. To the capped quantum dots was added EDC (1-ethyl-3-[3-dimethylaminopropyl] carbdiimide) and sulfoNHS (sulfo-N-hydroxysuccinimide) in 500-1000 times excess. The resulting solution was stirred at room temperature for 30 minutes. Mercaptoethanol was added to neutralize unreacted EDC at 20 mM concentration and stirred for 15 minutes. The entire solution was then added dropwise, with stirring, to a solution of lysine (large excess) in the same buffer; and the mixture was stirred for 2 hours at room temperature. Ethanolamine (30 mM) was added to quench the reaction; and the mixture was stirred for 30 minutes at room temperature or left overnight at 4°C. The solution was centrifuged to remove any precipitated solids, and then ultrafiltered through a 30kD MW centrifugal filter. The resultant concentrated, functionalized nanocrystals can be solubilized in an aqueous solution of choice. Once solubilized, the resulting solution can be stored in an amber vial under an inert gas to prevent flocculation.

In another embodiment, as also illustrated in FIG. 2, the functionalized nanocrystals comprised of a first layer comprising capping compound and a second layer comprising diaminocarboxylic acid, is further functionalized by the addition of affinity ligand. As an illustrative example, a protein (glycoprotein, peptide, lipoprotein, etc.) having a free carboxyl-reactive group (e.g., an amine group)

can be operably linked to the free carboxyl group of the diaminocarboxylic acid of the functionalized nanocrystals using methods known in the art. For example, an affinity ligand selected from the group consisting of avidin, a monoclonal antibody, an F'ab fragment, or a lectin (e.g., wheat germ agglutinin) may be operably linked using EDC and sulfo-NHS using the general methods as previously described herein. More particularly, EDC functions to activate at least one reactive functionality (e.g., a carboxylate) to catalyze its reaction with another reactive functionality such as the amine group of a protein. The functionalized nanocrystals (1 ml, 8.1×10^{-9} mol) were esterified by treatment with EDC (8.1×10^{-6} mol), followed by treatment with sulfo-NHS (8.9×10^{-6} mol) at ambient temperature in buffered aqueous solution (at about pH 7.4) for 30 minutes. 2-mercaptoethanol was added to the solution at a concentration of 20 mM, and the mixture was stirred for 15 minutes to quench any unreacted EDC. Using a lectin wheat germ agglutinin (WGA) as an exemplary affinity ligand, the nanocrystals were then contacted with WGA (8.1×10^{-9} mol in PBS, 1 mg/ml) with vigorous stirring, and the reaction mixture was stirred for 2 hours (e.g., conditions sufficient to form an amide bond between the EDC-activated carboxylates of the diaminocarboxylate layer and the amine groups on WGA in forming functionalized nanocrystals which are water-soluble and have lectin operably linked thereto). Ethanolamine was added at a concentration of 30 mM to quench the coupling reaction, and the reaction mixture was stirred for 30 minutes. The resulting solution was then filtered through a 30 kD molecular weight cutoff centrifugal filter to remove excess reagents. The concentrated material was then diluted

to 1 ml in buffer (e.g., PBS) or other suitable aqueous solution. Essentially, the same procedure can be used to operably link avidin, an antibody, or other affinity ligand having at least one free carboxyl-reactive group.

In illustrating an embodiment of a method of using the functionalized nanocrystals, it may be desirable to attach one or a plurality of oligonucleotides to the functionalized nanocrystals for subsequent use in a nucleic acid probe hybridization detection system. In one illustration of this embodiment, the functionalized nanocrystals comprise avidinylated, functionalized nanocrystals (e.g., (CdX) core/YZ shell, capped with the capping compound, coated with diaminocarboxylic acid that is operably linked to the capping compound, followed by addition of avidin which is operably linked to the diaminocarboxylic acid) which are then contacted with, and operably linked to, a plurality of molecules of the desired oligonucleotide, each of which contains one or more biotin molecules (including native biotin or a biotin derivative having avidin-binding activity; e.g., biotin dimers, biotin multimers, carbo-biotin, and the like). Preferably, the oligonucleotides are biotinylated at a single terminus of the strand. Using methods known to those skilled in the art, biotin molecules can be added to or incorporated in a nucleotide strand, and even localized to one terminus, such as by directing synthesis of the nucleotide strands with nucleotides and biotin-nucleotides, or by biotinylating the 5' aminogroup of the nucleotide with sulfo-NHS-biotin. Thus, by contacting avidinylated, functionalized nanocrystals with biotinylated oligonucleotides, formed is a functionalized nanocrystal having a plurality of oligonucleotides extending therefrom (e.g., through the

biotin-avidin binding, the plurality of oligonucleotides become operably linked to the functionalized nanocrystals). These functionalized nanocrystals may then be used as probes in a nucleic acid probe hybridization detection system using standard methods known to those skilled in the art.

EXAMPLE 2

In another embodiment of the functionalized nanocrystals according to the present invention, the functionalized nanocrystals comprise quantum dots with a first layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and a third layer comprising an amino acid. Functionalized nanocrystals comprising capping compound, and diaminocarboxylic acid may be produced using the methods outlined in Example 1, and FIG. 2 herein. These functionalized nanocrystals are further functionalized by the addition of another layer comprising an amino acid, such as illustrated in FIG. 3. FIG. 3 illustrates the addition of an additional layer of an amino acid wherein the amino acid comprises a diaminocarboxylic acid. In this illustration, the diaminocarboxylic acid molecules of the third layer can operably link, and crosslink, the free carboxyl groups of the diaminocarboxylic acid molecules of the second layer. However, it is noted that with each diaminocarboxylic acid layer added, the number of free functional groups for reaction to operably link with a subsequent carboxylic acid layer or affinity ligand is reduced. If, for example, an affinity ligand is to be operably coupled to diaminocarboxylic acid comprising a third layer, a reduction in the number of free functional groups for reaction with the affinity ligand may be desira-

ble, particularly if it is desired to operably link relatively fewer molecules of the affinity ligand to the functionalized nanocrystals (e.g., because of one or more of the size, chemical characteristics, and specificity of the affinity ligand, or substrate to which the affinity lignd binds). However, if a maximum number of affinity ligands is desired to be operably linked to the functionalized nanocrystals, it may be disadvantageous to use a third layer comprising an amino acid comprising a diaminocarboxylic acid. If a maximum number of affinity ligands is desirable, alternative embodiments include: (a) operably linking the affinity ligand to functionalized nanocrystals comprising quantum dots, the capping compound, and the diaminocarboxylic acid; or (b) operably linking a third layer (comprising an amino acid comprising monoaminocarboxylic acid operably linked to the diaminocarboxylic acid), and then operably link the affinity ligand to the functionalized nanocrystals via the free carboxyl group of the monoaminocarboxylic acid. Thus, various factors, such as the nature of the affinity ligand to be operably linked, may guide the choice of a carboxylic acid for a third layer in further functionalizing the nanocrystals according to the present invention.

As illustrated in FIG. 3, functionalized nanocrystals comprising quantum dots, capping compound, diaminocarboxylic acid, are mixed with EDC and sulfo-NHS in 500-1000 times excess. The resulting solution is stirred at room temperature for 30 minutes. Mercaptoethanol is added to neutralize unreacted EDC at 20 mM concentration and stirred for 15 minutes. The entire solution is then added dropwise, with stirring, to a solution of an amino acid compris-

ing a diaminocarboxylic acid (e.g., lysine in large excess) in the same buffer; and the mixture is stirred for 2 hours at room temperature. Ethanolamine (30 mM) is added to quench the reaction; and the mixture is stirred for 30 minutes at room temperature or left overnight at 4°C. The solution is centrifuged to remove and precipitate solids, and then ultrafiltered through a 30kD MW centrifugal filter. The resultant concentrated, functionalized nanocrystals can be solubilized in an aqueous solution of choice. This process can also be used to add a third layer comprising an amino acid comprising a monoaminocarboxylic acid rather than a diaminocarboxylic acid. In either case, functionalized nanocrystals comprising a third layer comprising an amino acid may be further functionalized by operably linking affinity ligand to the free amine reactive group(s) (or other free reactive groups) of the amino acid comprising the third layer using methods previously described herein. Using similar methods as those outlined above, diamino-carboxylic acid may be operably linked to a capping compound comprising mercapto-functionalized amine, and more particularly, by the use of a linker.

EXAMPLE 3

In a method of detection of a target substrate using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals are placed in contact with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand of the functionalized nanocrystals has binding specificity. Contact, and subsequent binding, between the affinity ligand of the functionalized nanocrystal and the sub-

strate, if present in the sample, in a detection system results in complexes comprising the functionalized nanocrystal-substrate which can emit a detectable signal for quantitation, visualization, or other form of detection. Upon formation of the complexes comprising the functionalized nanocrystal-substrate, the detectable signal emitted therefrom may be detected by first exposing the complexes formed in the detection system to a wavelength spectrum of light (visible, or UV, or a combination thereof) that is suitable for exciting the functionalized nanocrystals to emit a fluorescence peak. The peak is then detected, or detected and quantitated, by appropriate detection means (e.g., photodetector, filters, fluorescence microscope, and the like). Quantitation of the amount of substrate present is directly related to the intensity of the emitted fluorescence peak. As known to those skilled in the art of nanocrystals, the absorbance peak and fluorescence peak emissions depend on such factors which include, but are not limited to, the chemical nature, and size, of the functionalized nanocrystals. For example, functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 68.4 angstroms (A) may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color orange) at 609nm which may be detected using appropriate detection means. Functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 53.2 A may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color yellow) at 545 nm which may be detected using appropriate

detection means. Functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 46.6 A may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color green) at 522 nm which may be detected using appropriate detection means. Detection may be by detection means comprising a scanner or reader or other analytical instrument which can detect fluorescence peaks in the range of about 410 nm to about 750 nm; and, optionally (when more than one color is used in the detection system), distinguish between discrete fluorescence peaks within that range. In the class of nanocrystals used in the present invention, many sizes of which can be excited with a single excitation light source, resulting in many emissions of colors that can be detected simultaneously and distinctly. Thus, for example, it will be apparent to those skilled in the art that more than one target substrate may be detected in a detection system simultaneously by using more than one uniform size of functionalized nanocrystals; with each uniform size having an affinity ligand operably linked thereto which has a different binding specificity (hence can detect a different target substrate) than the affinity ligand operably linked to functionalized nanocrystals of a different uniform size. As will be apparent to one skilled in the art, the detection system may include, but is not limited to, one or more of an affinity assay (e.g, immunoassay such as an ELISA), fluorescent staining (e.g., immunofluorescence staining on a glass slide), flow cytometry, nucleic acid hybridization assay, molecular sorting (e.g., cell sorting by flow cytometry), and the like.

In one illustration of this embodiment, functionalized nanocrystals, comprising diaminocarboxylic acid which is operably linked to the capping compound, are further by the addition of affinity ligand, comprising lectin WGA (wheat germ agglutinin) which is operably linked to the diaminocarboxylic acid, by using the methods outlined herein in Example 1 ("WGA-labeled, functionalized nanocrystals). To a tube containing approximately 70,000 cells of Met-129 cancer cell line (chemically induced murine mammary carcinoma) was added 200 μl of the WGA-labeled, functionalized nanocrystals, and the mixture was then rotated gently on a platform mixer. Met-129 cells have one or more cell surface glycoproteins with either terminal N-acetylglucosamine residues or with terminal sialic acid residues (e.g., mucin) which may be reactive with WGA. After 10 minutes, a drop of the mixture was placed on a microscope slide, and covered with a coverslip. Examination of the sample with a fluorescence microscope revealed that the Met-129 cells aggregated together, with the outlines of the cells clearly visible by fluorescent staining with the WGA-labeled, functionalized nanocrystals. There was very little background fluorescence remaining in the reaction media. After 30 minutes, another sample was examined, and again at 2 hours. Both of the latter samples showed agglutination of the cells, with fluorescent staining of the outside cell walls by the WGA-labeled, functionalized nanocrystals.

As a negative control for the staining mediated by the WGA-labeled, functionalized nanocrystals, unlabeled functionalized nanocrystals were added to a tube containing Met-129 cells. At 10 minutes and 30 minutes, a very low level of non-specific staining of cells was observed. In a

positive control reaction, WGA-labeled with Oregon Green fluorescent dye was added to a tube containing Met-129 cells. At each sample time, the cells were observed as large, brightly stained aggregates. However, the cell media retained a high level of background fluorescence.

The foregoing description of the specific embodiments of the present invention have been described in detail for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying, current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept, and therefore such modifications and/or adaptations are intended to be within the meaning and scope of the appended claims.

What is claimed:

1. A water-soluble, functionalized nanocrystal comprising: a quantum dot having a core and a shell; a capping compound operably linked to the quantum dot; and a diaminocarboxylic acid which is operably linked to the capping compound.

2. The water-soluble, functionalized nanocrystal according to claim 1, wherein the core comprises CdSe.

3. The water-soluble, functionalized nanocrystal according to claim 1, wherein the shell comprises ZnS.

4. The water-soluble, functionalized nanocrystal according to claim 1, wherein the capping compound comprises mercaptocarboxylic acid.

5. The water-soluble, functionalized nanocrystal according to claim 1, wherein the diaminocarboxylic acid forms a coating over the capping compound.

6. The water-soluble, functionalized nanocrystal according to claim 1, wherein and the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

7. The water-soluble, functionalized nanocrystal according to claim 1, further comprising affinity ligand which is operably linked to the diaminocarboxylic acid.

8. The water-soluble, functionalized nanocrystal according to claim 7, wherein the affinity ligand forms a layer over the diaminocarboxylic acid.

9. The water-soluble, functionalized nanocrystal according to claim 7, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

10. A water-soluble, functionalized nanocrystal comprising: a quantum dot; a capping compound operably linked to the quantum dot; diaminocarboxylic acid which is operably linked to the capping compound; and amino acid which is operably linked to the diaminocarboxylic acid.

11. The water-soluble, functionalized nanocrystal according to claim 10, wherein the core comprises CdSe.

12. The water-soluble, functionalized nanocrystal according to claim 10, wherein the shell comprises ZnS.

13. The water-soluble, functionalized nanocrystal according to claim 10, wherein the capping compound comprises mercaptocarboxylic acid.

14. The water-soluble, functionalized nanocrystal according to claim 10, wherein the amino acid forms a coating over the diaminocarboxylic acid.

15. The water-soluble, functionalized nanocrystal according

to claim 10, wherein the amino acid comprises a diaminocarboxylic acid.

16. The water-soluble, functionalized nanocrystal according to claim 15, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

17. The water-soluble, functionalized nanocrystal according to claim 10, wherein the amino acid comprises a monoaminocarboxylic acid, and the monoaminocarboxylic acid is selected from the group consisting of glycine, serine, threonine, cysteine, β-alanine, homoserine, γ-aminobutyric acid, and homocysteine.

18. The water-soluble, functionalized nanocrystal according to claim 10, further comprising affinity ligand which is operably linked to the amino acid.

19. The water-soluble, functionalized nanocrystal according to claim 18, wherein the affinity ligand forms a layer over the amino acid.

20. The water-soluble, functionalized nanocrystal according to claim 18, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

21. A water-soluble, functionalized nanocrystal comprising: a quantum dot; a capping compound operably linked to the quantum dot, wherein the capping compound comprises a mercapto-functionalized amine; and diaminocarboxylic acid which is operably linked to the capping compound.

22. The water-soluble, functionalized nanocrystal according to claim 21, wherein a linker is used to operably link the diaminocarboxylic acid to the capping compound.

23. The water-soluble, functionalized nanocrystal according to claim 21, wherein the diaminocarboxylic acid forms a coating over the capping compound.

24. The water-soluble, functionalized nanocrystal according to claim 21, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

25. The water-soluble, functionalized nanocrystal according to claim 21, further comprising affinity ligand which is operably linked to the diaminocarboxylic acid.

26. The water-soluble, functionalized nanocrystal according to claim 25, wherein the affinity ligand forms a layer over the diaminocarboxylic acid.

27. The water-soluble, functionalized nanocrystal according to claim 25, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

28. The water-soluble, functionalized nanocrystal according to claim 21, further comprising an amino acid which is operably linked to the diaminocarboxylic acid.

29. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid forms a layer over the diaminocarboxylic acid.

30. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid comprises a diaminocarboxylic acid.

31. The water-soluble, functionalized nanocrystal according to claim 30, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

32. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid comprises a monoaminocarboxylic acid, and the monoaminocarboxylic acid is selected from the group consisting of glycine, serine,

threonine, cysteine, β-alanine, homoserine, γ-aminobutyric acid, and homocysteine.

33. The water-soluble, functionalized nanocrystal according to claim 28, further comprising affinity ligand which is operably linked to the amino acid.

34. The water-soluble, functionalized nanocrystal according to claim 33, wherein the affinity ligand forms a layer over the amino acid.

35. The water-soluble, functionalized nanocrystal according to claim 33, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

36. A method of using the water-soluble, functionalized nanocrystal according to claim 7 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

37. The method according to claim 36, wherein the presence of the substrate is detected, and further comprises quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

38. The method according to claim 36, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

39. A method of using the water-soluble, functionalized nanocrystal according to claim 18 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

40. The method according to claim 39, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

41. The method according to claim 39, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

42. A method of using the water-soluble, functionalized nanocrystal according to claim 25 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

43. The method according to claim 42, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

44. The method according to claim 42, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

45. A method of using the water-soluble, functionalized nanocrystal according to claim 33 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

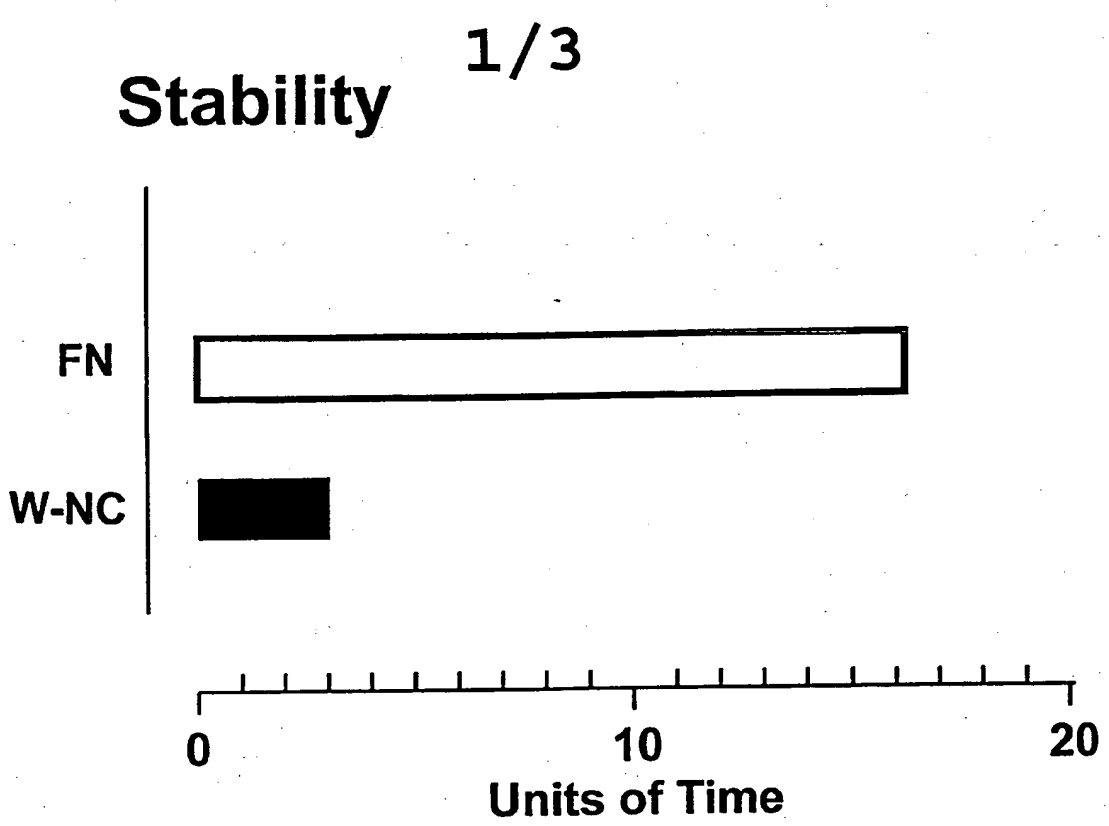
(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

46. The method according to claim 45, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

47. The method according to claim 45, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.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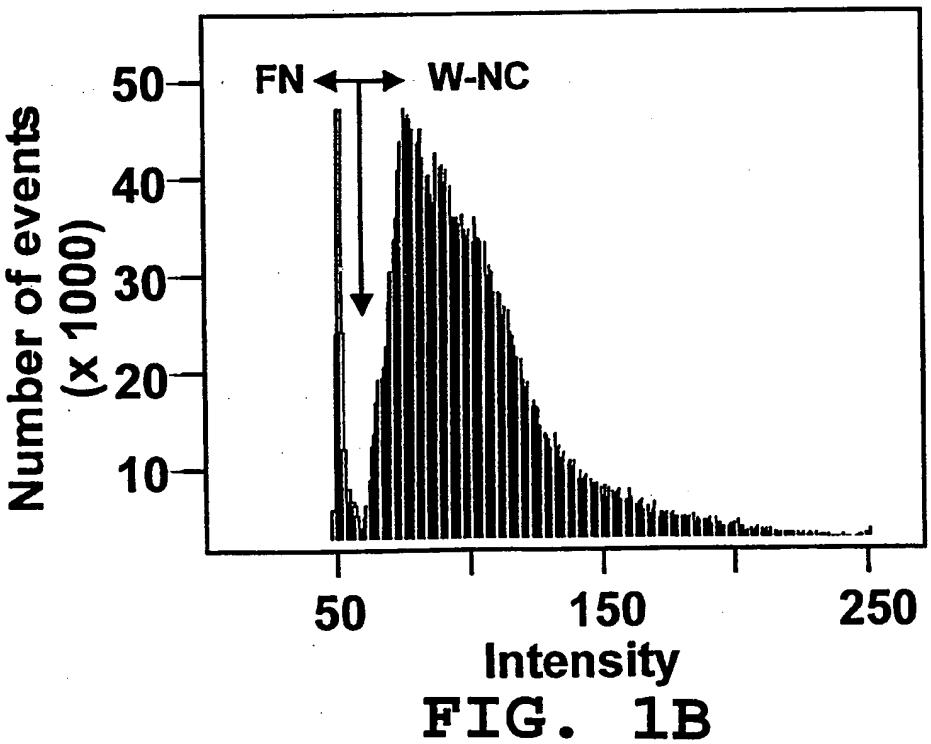
W-NC
0
10
20
Units of Time

FIG. 1A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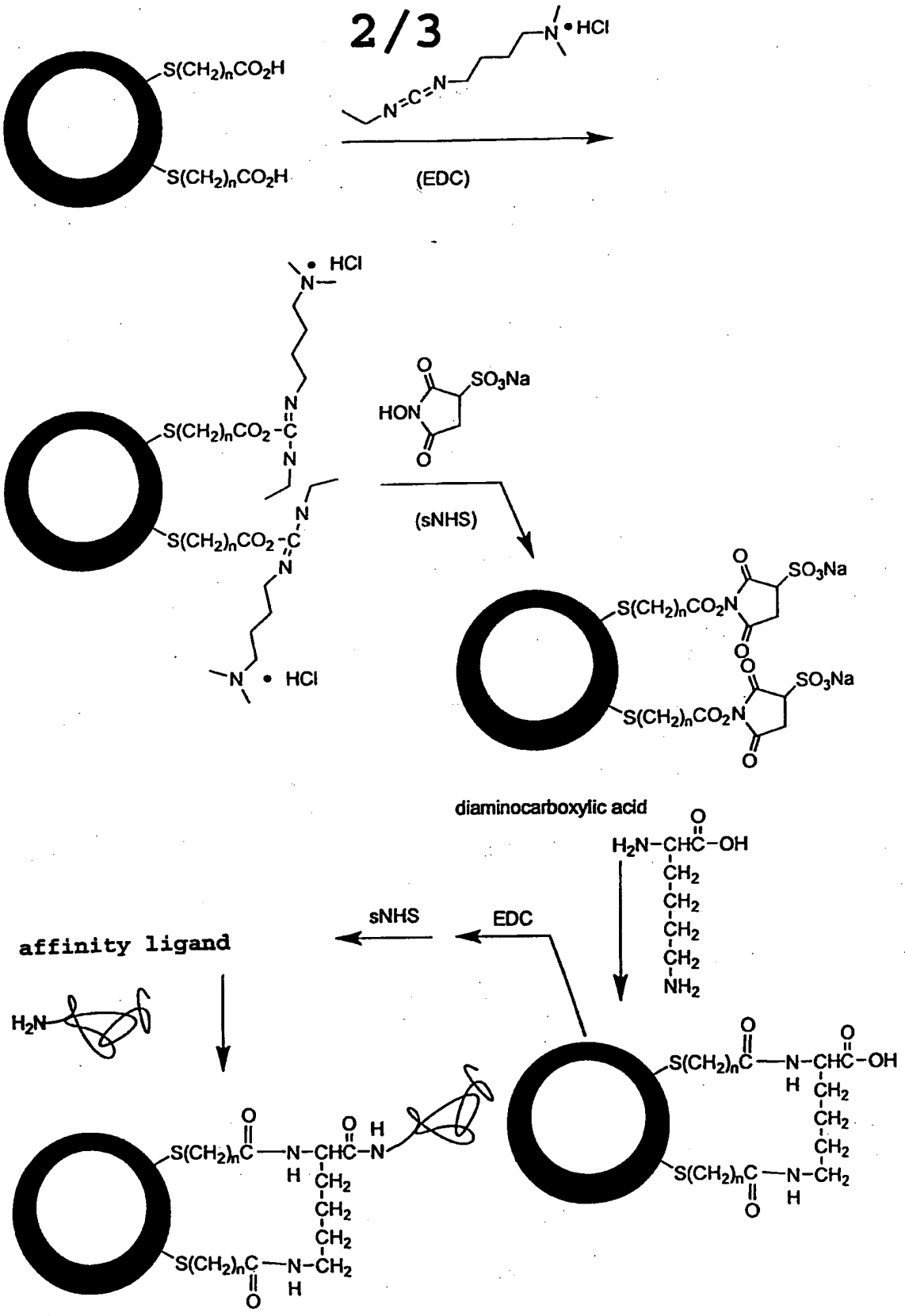
40
30
20
10
Number of events
(x 1000)
50
150
250
Intensity

FIG. 1B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S(CH2)nCO2H
N=C=N
N • HCl
(EDC)
• HCl
N
HON
O
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S(CH2)nCO2
(sNHS)
diaminocarboxylic acid
H2N–CHC–OH
CH2
NH2
sNHS
EDC
affinity ligand
H2N
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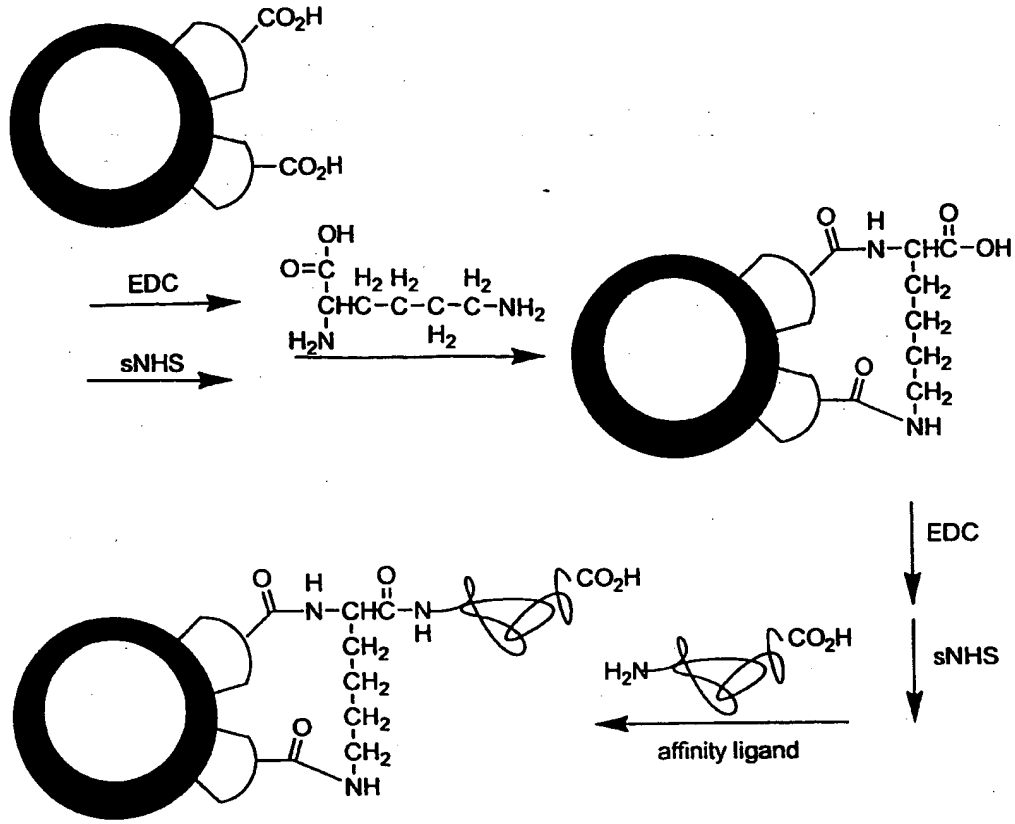
H
S(CH2)nC–N–CH2
S(CH2)nC–N–CHC–OH

FIG. 2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OH
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H2
H2 H2
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N-CHC-OH
CH2
CH2
CH2
CH2
O
NH
EDC
sNHS
CO2H
H2N
CO2H
affinity ligand
O
H
N-CHC-N
H
CH2
CH2
CH2
CH2
O
NH

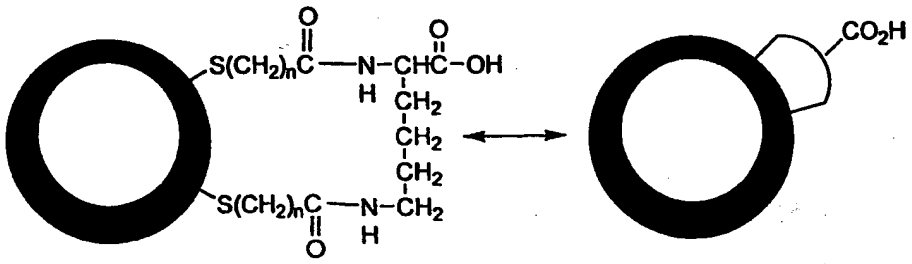
O
S(CH2)nC
N-CHC-OH
H
CH2
CH2
CH2
S(CH2)nC
N-CH2
O
H
CO2H

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26487

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/307, 361C 361R, 432R, 459.1; 356/317; 378/45, 48; 424/490; 436/546; 428/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST 1.2, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,525,377 A (GALLAGHER et al) 11 June 1996, entire document.	1-47
A	US 5,882,779 A (LAWANDY) 16 March 1999, entire document.	1-47
A	US 5,908,608 A (LAWANDY et al) 01 June 1999, entire document.	1-47
A	US 5,990,479 A (WEISS et al) 23 November 1999, entire document.	1-47
A	JP 11087689 A (EGAWA) 30 March 1999, entire document.	1-47
A	JP 11154771 A (FUJII) 08 June 1999, entire document.	1-47

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search	Date of mailing of the international search report
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CORRECTED VERSION* | CORRECTED VERSION**

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(54) Title: FUNCTIONALIZED NANOCRYSTALS AND THEIR USE IN DETECTION SYSTEMS

(57) Abstract

Provided are compositions comprising water–soluble, functionalized nanocrystals. The water–soluble functionalized nanocrystals comprise quantum dots capped with a layer of a capping compound, and further comprise, by operably linking and in a successive manner, one or more additional compounds. Preferably, an additional compound comprises diaminocarboxylic acid which is operatively linked to the capping compound, and may further comprise an amino acid, and affinity ligand, or a combination thereof. Also provided are methods of using the functionalized nanocrystals having an affinity ligand to detect the presence or absence of a target substrate in a sample by contacting the functionalized nanocrystals with the sample so that complexes are formed between the functionalized nanocrystals and substrate, if the substrate is present; exposing the complexes in the detection system to an excitation light source, and detecting the emitted fluorescence peak.

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FUNCTIONALIZED NANOCRYSTALS AND THEIR USE IN DETECTION SYSTEMS

FIELD OF INVENTION

This invention relates to novel compositions comprising functionalized nanocrystals. More particularly, the present invention relates to water-soluble nanocrystals which have a coat comprising a capping compound, and one or more additional compounds successively overlayered onto the capped nanocrystal. The present invention also relates to the use of the functionalized nanocrystals for providing a detectable signal in detection systems in which the nanocrystals are employed.

BACKGROUND OF THE INVENTION

Nonisotopic detection systems have become a preferred mode in scientific research and clinical diagnostics for the detection of biomolecules using various assays including flow cytometry, nucleic acid hybridization, DNA sequencing, nucleic acid amplification, immunoassays, histochemistry, and functional assays involving living cells. In particular, while fluorescent organic molecules such as fluoroscein and phycoerythrin are used frequently in detection systems, there are disadvantages in using these molecules in combination. For example, each type of fluorescent molecule typically requires excitation with photons of a different wavelength as compared to that required for another type of fluorescent molecule. However, even when a single light source is used to provide a single excitation wavelength (in view of the spectral line width), often there is insufficient spectral spacing between the emission optima of different fluorescent molecules to permit individual and

quantitative detection without substantial spectral overlap. Further, currently available nonisotopic detection systems typically are limited in sensitivity due to the finite number of nonisotopic molecules which can be used to label a biomolecule to be detected.

Semiconductor nanocrystals ("quantum dots") are known in the art. Generally, quantum dots can be prepared which result in relative monodispersity (e.g., the diameter of the core varying approximately less than 10% between quantum dots in the preparation), as has been described previously. Examples of quantum dots are known in the art to have a core selected from the group consisting of CdSe, CdS, and CdTe (collectively referred to as "CdX").

CdX quantum dots have been passivated with an inorganic coating ("shell") uniformly deposited thereon. Passivating the surface of the core quantum dot can result in an increase in the quantum yield of the fluorescence emission, depending on the nature of the inorganic coating. The shell which is used to passivate the quantum dot is preferably comprised of YZ wherein Y is Cd or Zn, and Z is S, or Se. Quantum dots having a CdX core and a YZ shell have been described in the art. However, the above described quantum dots, passivated using an inorganic shell, have only been soluble in organic, non-polar (or weakly polar) solvents.

To make quantum dots useful in biological applications, it is desirable that the quantum dots are water-soluble. "Water-soluble" is used herein to mean sufficiently soluble or suspendable in a aqueous-based solution, such as in water or water-based solutions or buffer solutions, including those used in biological or molecular

detection systems as known by those skilled in the art. Typically, CdX core/YZ shell quantum dots are over-coated with trialkylphosphine oxide, with the alkyl groups most commonly used being butyl and octyl. One method to make the CdX core/YZ shell quantum dots water-soluble is to exchange this overcoating layer with a coating which will make the quantum dots water-soluble. For example, a mercaptocarboxylic acid may be used to exchange with the trialkylphosphine oxide coat. Exchange of the coating group is accomplished by treating the water-insoluble quantum dots with a large excess of neat mercaptocarboxylic acid. Alternatively, exchange of the coating group is accomplished by treating the water-insoluble quantum dots with a large excess of mercaptocarboxylic acid in $CHCl_3$ solution. The thiol group of the new coating molecule forms Cd (or Zn)-S bonds, creating a coating which is not easily displaced in solution. Another method to make the CdX core/YZ shell quantum dots water-soluble is by the formation of a coating of silica around the dots. An extensively polymerized polysilane shell imparts water solubility to nanocrystalline materials, as well as allowing further chemical modifications of the silica surface. However, depending on the nature of the coating group, quantum dots which have been reported as water-soluble may have limited stability in an aqueous solution, particularly when exposed to air (oxygen) and/or light. More particularly, oxygen and light can cause the molecules comprising the coating to become oxidized, thereby forming disulfides which destabilize the attachment of the coating molecules to the shell. Thus, oxidation may cause the coating molecules to migrate away from the surface of the nanocrystals, thereby exposing the surface of the

nanocrystals in resulting in "destabilized nanocrystals". Destabilized nanocrystals form aggregates when they interact together, and the formation of such aggregates eventually leads to irreversible flocculation of the nanocrystals (e.g., see FIG. 1A).

Thus, there remains a need for a semiconductor nanocrystal which (a) is water-soluble; (b) is functionalized to enhance stability in aqueous solutions; (c) is a class of semiconductor nanocrystals that may be excited with a single wavelength of light resulting in detectable luminescence emissions of high quantum yield and with discrete luminescence peaks; and (d) is functionalized so as to be both water-soluble, and able to bind ligands, molecules, or probes of various types for use in an aqueous-based environment.

SUMMARY OF THE INVENTION

The present invention provides a composition comprising functionalized nanocrystals for use in non-isotopic detection systems. The composition comprises quantum dots (capped with a layer of a capping compound) that are water-soluble and functionalized by operably linking, in a successive manner, one or more additional compounds. In a preferred embodiment, the one or more additional compounds form successive layers over the nanocrystal. More particularly, the functionalized nanocrystals comprise quantum dots capped with the capping compound, and comprise a coating (a plurality of molecules comprising) diaminocarboxylic acid which is operatively linked to the capping compound. Thus, the functionalized nanocrystals may have a first layer comprising the capping compound, and a

second layer comprising diaminocarboxylic acid; and may further comprise one or more successive layers including a layer of amino acid, a layer of affinity ligand, or multiple layers comprising a combination thereof. The composition comprises a class of quantum dots that can be excited with a single wavelength of light resulting in a detectable luminescence emissions of high quantum yield and with discrete luminescence peaks.

In a method of detection of a target substrate using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals are further functionalized by binding an affinity ligand thereto. The resultant functionalized nanocrystals are placed in contact with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity. Contact, and subsequent binding, between the affinity ligand of the functionalized nanocrystals and the substrate, if present in the sample, results in a complex comprising the functionalized nanocrystal-substrate which can emit a detectable signal for quantitation, visualization, or other form of detection.

The above and other objects, features, and advantages of the present invention will be apparent in the following Detailed Description of the Invention when read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** is a bar graph comparing the stability of capped quantum dots ("W-SN") to the stability of functionalized nanocrystals ("FN") under oxidizing conditions.

FIG. **1B** is a bar graph comparing the non-specific binding of capped quantum dots ("W-SN") to the non-specific binding of functionalized nanocrystals ("FN").

FIG. **2** is a schematic illustrating chemically modifying a water-soluble quantum containing a layer of a capping compound to further comprise a layer of a diaminocarboxylic acid, and a layer of an affinity ligand (e.g., avidin).

FIG. **3** is a schematic illustrating chemically modifying a water soluble quantum dot containing a layer of a capping compound to further comprise a layer of a diaminocarboxylic acid, an additional layer of a diaminocarboxylic acid, and a layer of an affinity ligand.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

By the term "substrate" is meant, for the purposes of the specification and claims to refer to a molecule of an organic or inorganic nature, the presence and/or quantity of which is being tested for; and which contains a molecular component (domain or sequence or epitope or portion or chemical group or determinant) for which the affinity ligand has binding specificity. The molecule may include, but is not limited to, a nucleic acid molecule, protein, glycoprotein, eukaryotic or prokaryotic cell, lipoprotein, peptide, carbohydrate, lipid, phospholipid, aminoglycans, chemical messenger, biological receptor, structural component, metabolic product, enzyme, antigen, drug, therapeutic, toxin, inorganic chemical, organic chemical, and the like. The substrate may be *in vivo*, *in vitro*, *in*

situ, or *ex vivo*. A preferred substrate may be used to the exclusion of a substrate other than the preferred substrate.

By the term "affinity ligand" is meant, for purposes of the specification and claims, to mean a molecule which has binding specificity and avidity for a molecular component of, or associated with, a substrate. In general, affinity ligands are known to those skilled in the art to include, but are not limited to, lectins or fragments (or derivatives) thereof which retain binding function; monoclonal antibodies ("mAb", including chimeric or genetically modified monoclonal antibodies (e.g., "humanized")); peptides; aptamers; nucleic acid molecules (including, but not limited to, single stranded RNA or single-stranded DNA, or single-stranded nucleic acid hybrids); avidin, or streptavidin, or avidin derivatives; and the like. The invention may be practiced using a preferred affinity ligand (e.g., a lectin) to the exclusion of affinity ligands other than the preferred affinity ligand. The term "monoclonal antibody" is also used herein, for purposes of the specification and claims, to include immunoreactive fragments or derivatives derived from a mAb molecule, which fragments or derivatives retain all or a portion of the binding function of the whole mAb molecule. Such immunoreactive fragments or derivatives are known to those skilled in the art to include $F(ab')_2$, Fab', Fab, Fv, scFV, Fd' and Fd fragments. Methods for producing the various fragments or derivatives from mAbs are well known in the art. For example, $F(ab')_2$ can be produced by pepsin digestion of the monoclonal antibody, and Fab' may be produced by reducing the disulfide bridges of $F(ab')_2$ fragments. Fab fragments can be produced by papain digestion of the monoclonal antibody, whereas Fv can be prepared

according to methods described in U.S. Patent No. 4,642,334. Single chain antibodies can be produced as described in U.S. Patent No. 4,946,778. The construction of chimeric antibodies is now a straightforward procedure in which the chimeric antibody is made by joining the murine variable region to a human constant region. Additionally, "humanized" antibodies may be made by joining the hypervariable regions of the murine monoclonal antibody to a constant region and portions of variable region (light chain and heavy chain) sequences of human immunoglobulins using one of several techniques known in the art. Methods for making a chimeric non-human/human mAb in general are known in the art (see, e.g., U.S. Patent No. 5,736,137). Aptamers can be made using methods described in U.S. Patent No. 5,789,157. Lectins, and fragments thereof, are commercially available. Lectins are known to those skilled in the art to include, but are not limited to, one or more of Aleuria aurantia lectin, Amaranthus caudatus lectin, Concanavalin A, Datura stramonium lectin, Dolichos biflorus agglutinin, soybean agglutinin, Erythrina cristagalli lectin, Galanthus nivalis lectin, Griffonia simplicifolia lectins, Jacalin, Macckia amurensis lectins, Maclura pomifera agglutinin, Phaeolepiota aurea lectins 1 and 2, Phaseolus vulgaris lectins, Ricin A, Moluccella laevis lectin, peanut agglutinin, Bauhinia purpurea agglutinin, Ricinus communis agglutinins, Sambucus nigra lectin, Vicia villosa agglutinin, Sophora japonica agglutinin, Caragana arborescens agglutinin, Helix aspersa agglutinin, Limax flavus lectin, limulin, wheat germ agglutinin, and Ulex europaeus agglutinin. A preferred affinity ligand may be used to the exclusion of an affinity ligand other than the preferred affinity ligand.

By the term "operably linked" is meant, for purposes of the specification and claims to refer to fusion or bond or an association of sufficient stability to withstand conditions encountered in a method of detection, between a combination of different molecules such as, but not limited to, between the quantum dot and a capping compound, between a capping compound and a diaminocarboxylic acid, between a diaminocarboxylic acid and a diaminocarboxylic acid, between a diaminocarboxylic acid and an affinity ligand, between a diaminocarboxylic acid and an amino acid, and between an amino acid and an affinity ligand, and a combination thereof. As known to those skilled in the art, and as will be more apparent by the following embodiments, there are several methods and compositions in which two or more molecules may be operably linked utilizing reactive functionalities. Reactive functionalities include, but are not limited to, bifunctional reagents/linker molecules, biotin, avidin, free chemical groups (e.g., thiol, or carboxyl, hydroxyl, amino, amine, sulfo, etc.), and reactive chemical groups (reactive with free chemical groups). A preferred reactive functionality may be used to the exclusion of a reactive functionality other than the preferred reactive functionality.

By the term "linker" is meant, for purposes of the specification and claims to refer to a compound or moiety that acts as a molecular bridge to operably link two different molecules, wherein one portion of the linker is operably linked to a first molecule, and wherein another portion of the linker is operably linked to a second molecule. The two different molecules may be linked to the linker in a step-wise manner. There is no particular size or

content limitations for the linker so long as it can fulfill its purpose as a molecular bridge. Linkers are known to those skilled in the art to include, but are not limited to, chemical chains, chemical compounds, carbohydrate chains, peptides, haptens, and the like. The linkers may include, but are not limited to, homobifunctional linkers and heterobifunctional linkers. Heterobifunctional linkers, well known to those skilled in the art, contain one end having a first reactive functionality to specifically link a first molecule, and an opposite end having a second reactive functionality to specifically link to a second molecule. As illustrative examples, to operably link a hydroxyl group of a polynucleotide strand to an amino group of a diaminocarboxylic acid, the linker may have: a carboxyl group to form a bond with the polynucleotide, and a carboxyl group to form a bond with the diaminocarboxylic acid. Heterobifunctional photo-reactive linkers (e.g., phenylazides containing a cleavable disulfide bond) are known in the art. For example, a sulfosuccinimidyl-2-(p-azido salicylamido) ethyl-1,3'-dithiopropionate contains a N-hydroxy-succinimidyl group reactive with primary amino groups, and the phenyl-azide (upon photolysis) reacts with any amino acids. The linker may further comprise a protective group which blocks reactivity with a functional group on the linker which is used to react with and bind to a molecule to be linked. A deprotection reaction may involve contacting the linker to one or more conditions and/or reagents which removes the protective group, thereby exposing the functional group to interact with the molecule to be linked. Depending on the nature of the protective group, deprotection can be achieved by various methods known in the art, including, but not

limited to photolysis, acidolysis, hydrolysis, and the like. Depending on such factors as the molecules to be linked, and the conditions in which the method of detection is performed, the linker may vary in length and composition for optimizing such properties as flexibility, stability, and resistance to certain chemical and/or temperature parameters. For example, short linkers of sufficient flexibility include, but are not limited to, linkers having from 2 to 10 carbon atoms. A preferred linker may be used to the exclusion of a linker other than the preferred linker.

By the term "diaminocarboxylic acid" is meant, for purposes of the specification and claims to refer to an amino acid that has two free amine groups. The amino acid may be a naturally occurring amino acid, a synthetic amino acid, a modified amino acid, an amino acid derivative, and an amino acid precursor (e.g., citrulline and ornithine are intermediates in the synthesis of arginine). In a preferred embodiment, the diaminocarboxylic acid contains neutral (uncharged) polar functional groups which can hydrogen bond with water, thereby making the diaminocarboxylic acid (and the quantum dot to which it is made a part of) relatively more soluble in aqueous solutions containing water than those with nonpolar functional groups. Exemplary diaminocarboxylic acids include, but are not limited to, lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, and synthetic diaminocarboxylic acids such as 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid. A preferred diaminocarboxylic acid may be used to the exclu-

sion of a diaminocarboxylic acid other than the preferred diaminocarboxylic acid.

By the term "amino acid" is meant, for purposes of the specification and claims to refer to a molecule that has at least one free amine group and at least one free carboxyl group. The amino acid may have more than one free amine group, or more than one free carboxyl group, or may further comprise one or more free chemical reactive groups other than an amine or a carboxyl group (e.g., a hydroxyl, a sulfhydryl, etc.). The amino acid may be a naturally occurring amino acid, a synthetic amino acid, a modified amino acid, an amino acid derivative, and an amino acid precursor. The amino acid may further be selected from the group consisting of a monoaminocarboxylic acid, and a diaminocarboxylic acid. In a preferred embodiment, the monoaminocarboxylic acid contains one or more neutral (uncharged) polar functional groups which can hydrogen bond with water, thereby making the monoaminocarboxylic acid (and the quantum dot to which it is made a part of) relatively more soluble in aqueous solutions containing water than those with non-polar functional groups. Exemplary monoaminocarboxylic acids include, but are not limited to, glycine, serine, threonine, cysteine, β-alanine, homoserine, and γ-aminobutyric acid. A preferred amino acid may be used to the exclusion of an amino acid other than the preferred amino acid.

By the term "capping compound" is meant, for purposes of the specification and claims to refer to a compound having the formula $HS(CH_2)_nX$, wherein X is a carboxylate (carboxylic moiety); or the formula $HS(CH_2)_nYX$, wherein X is a carboxylate and Y is an amine; as will be more apparent

from the following descriptions. "n" is a number in the range of from 1 to about 20, and preferably greater than 4. The thiol group of the capping compound forms Cd (or Zn)-S bonds (depending on whether the shell is Cd or Zn), creating a layer which is not easily displaced in solution. Additionally, the carboxylic acid moiety of the capping compound imparts some water solubility to the quantum dots. Exemplary capping compounds according to the present invention include, but are not limited to, mercaptocarboxylic acid, or mercaptofunctionalized amines (e.g., aminoethanethiol-HCl, homocysteine, or 1-amino-2-methyl-2-propanethiol-HCl). A preferred capping compound may be used to the exclusion of a capping compound other than the preferred capping compound.

The present invention provides compositions which can be used to generate a detectable signal comprising a light emission (e.g., fluorescence emission) of high quantum yield, thereby considerably improving the sensitivity of a non-isotopic detection system. According to the present invention, functionalized nanocrystals comprise quantum dots (core and shell) which comprises a first additional layer or coating comprising a capping compound, and a second layer or coating comprising diaminocarboxylic acid. In another embodiment of the present invention, functionalized nanocrystals comprise quantum dots which comprise a first layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and an addition comprising affinity ligand (one or more molecules of affinity ligand). In another embodiment of the present invention, functionalized nanocrystals comprise quantum dots which comprising a first

layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and a third layer comprising amino acid. In yet another embodiment of the present invention, functionalized nanocrystals comprise quantum dots (core and shell) which comprise a first layer or coating comprising the capping compound, a second layer comprising diaminocarboxylic acid, a third layer comprising amino acid, and wherein the third layer has operably linked thereto one or more molecules of affinity ligand. In each of the embodiments, the component of each successive layer is operably linked to the component of any contacting layer, as will be more apparent from the figures and following description.

In one embodiment of a method for using the functionalized nanocrystals according to the present invention, the functionalized nanocrystal comprises quantum dots, the capping compound, diaminocarboxylic acid, and operably linked to diaminocarboxylic acid is one or more molecules of affinity ligand. The functionalized nanocrystals are first contacted with a sample under conditions suitable for the nanocrystals to contact and bind, via the affinity ligand portion, the substrate, if present, in the sample being analyzed for the presence or absence of the substrate. Alternatively, the functionalized nanocrystals may comprise quantum dots, the capping compound, diaminocarboxylic acid, amino acid, and affinity ligand operably lnked to the amino acid.

In another embodiment of a method for using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals comprise quantum dots, a coating of capping compound, and a coating comprising

diaminocarboxylic acid. The user may then operably link the desired affinity ligand to the diaminocarboxylic acid of the functionalized nanocrystal using methods known in the art. Alternatively, the functionalized nanocrystals may comprise quantum dots, a coating comprising the capping compound, a coating comprising diaminocarboxylic acid, and a coating comprising an amino acid; and the user may then operably link the desired affinity ligand to the amino acid of the functionalized nanocrystal using methods known in the art.

EXAMPLE 1

In one preferred embodiment, the composition according to the present invention comprises quantum dots which are capped by the addition of a layer comprising a capping compound, and more preferably a capping compound having the formula $HS(CH_2)_nX$, (wherein X is a carboxylic moiety), and comproises one or more successive layers comprising diaminocarboxylic acid, amino acid, or a combination thereof. Desirable features of the functionalized nanocrystals according to the present invention are that (a) can be excited with a single excitation light source, (b) when excited, emit a detectable light emission (e.g., fluorescence emission) of high quantum yield (e.g., a single quantum dot having at a fluorescence intensity at least a log greater than that of conventional fluorescent dye molecules), (c) have a light emission having a discrete fluorescence peak, and (d) are water-soluble. The functionalized nanocrystals typically should comprise a quantum dot particle of substantially uniform size of less than 100 Angstroms, and preferably have a substantially uniform size in the range of sizes of from about 2 nm to

about 10 nm (diameter). Preferred quantum dots used in the production of functionalized nanocrystals are comprised of a core of CdSe passivated with ZnS.

In this embodiment is illustrated the production of the functionalized nanocrystals. Exemplary quantum dots comprise a CdSe core, and a ZnS shell, "(CdSe)ZnS". TOPO capped CdSe were produced by placing TOPO (5g) in a vessel, and dried at 150°C for 1 hour under vacuum. The vessel was then backfilled with argon and heated to 300°C. In a controlled environment, $CdMe_2$ (7.2 µl , 0.1 mmol) and 1 M trioctylphosphine-Se solution (90 µl , 0.09 mmol) and trioctylphosphine (5 ml) were mixed, and then placed into an injector. This mixture was added to the TOPO in a reaction vessel, previously removed from the heat, in a single continuous injection with vigorous stirring, thereby resulting in the temperature decreasing to about 180°C. The reaction vessel was then subjected to heat to raise the temperature 5°C every 10 minutes. Aliquots may be removed from the reaction vessel at various time intervals (5 to 10 minutes) to monitor the increase in size of nanocrystals over time, by the observation of the absorption spectra. The temperature may be changed, or the reaction halted, upon reaching nanocrystals of the desired characteristics. For example, the reaction vessel was cooled to about 60°C, 40 ml of methanol was added to cause the nanocrystals to flocculate. After centrifugation, a brightly colored liquid layer of nanocrystals dissolved in trioctylphosphine remained. The methanol/TOPO layer was decanted off, and pyridine (10 ml) was added to the nanocrystal solution and allowed to stand for at least one hour. The nanocrystals were then precipitated as a powder by addition of hexanes, and separated

by centrifugation. The powder was washed once more with hexanes, then dissolved in 30 ml pyridine, and centrifuged to remove any reaction byproducts.

To prepare (CdSe)ZnS nanocrystals, the pyridine solution (30 ml) was placed in a reaction vessel, rigorously degassed with an inert gas (e.g., argon), and refluxed for one hour before adjusting the temperature to approximately 100°C. Equimolar amounts of diethyl zinc (zinc source) and hexamethyldisilathiane (sulfide source) were dissolved in trioctylphosphine (2-4 ml) in a controlled environment (glove box) and loaded into an injector. A reaction vessel containing the CdSe dots dispersed in pyridine was heated under an atmosphere of argon, and the Zn and S were added dropwise, via the injector, with vigorous stirring of the mixture for 5-10 minutes. The mixture was left stirring for several hours. After cooling, the pyridine solution was centrifuged to remove any insoluble material. The over-coated nanocrystals were stored in this solution to ensure that the surface of the nanocrystals remained passivated with pyridine.

To prepare nanocrystals which are capped, the pyridine overcoating of the (CdX) core/YZ shell nanocrystals were exchanged with a capping compound which contributes to the water-solubility of the resultant nanocrystals. For example, a capping compound comprising mercaptocarboxylic acid may be used to exchange with the pyridine overcoat. Exchange of the coating group is accomplished by treating the water-insoluble, pyridine-capped quantum dots with a large excess of neat mercapto-carboxylic acid. To accomplish this, the pyridine-capped (CdSe)ZnS quantum dots were precipitated with hexanes, and then isolated by centri-

fugation. The residue was dissolved in neat mercaptoacetic acid, with a few drops of pyridine added, if necessary, to form a transparent solution. The solution is allowed to stand at room temperature for at least six hours. Longer incubation times lead to increased substitution by the thiol. Overnight incubations are ideal. Chloroform is added to precipitate the nanocrystals and wash away excess thiol. The nanocrystals were isolated by centrifugation, washed once more with chloroform, and then washed with hexanes. The residue was briefly dried with a stream of argon. The resultant nanocrystals, coated with the capping compound, showed some solubility in water or other aqueous solutions. The nanocrystals, in an aqueous solution, were centrifuged once more, filtered through a 0.2 μm filter, degassed with argon, and stored in an amber vial. Failure to protect the nanocrystals, in solution, from air and light leads to rapid, irreversible flocculation.

Thus, single-site attachment of the capping compound (a mercaptocarboxylic acid; e.g., mercaptoacetic acid, mercaptopropionic acid, mercaptoundecanoic acid, etc.) suffers from limited stability in aqueous solution in the presence of water when exposed to air (oxygen) and light. It was found that by functionalizing the nanocrystal by adding a coating of diaminocarboxylic acid, resulted in significant enhancement of solubility and stability of the resultant functionalized nanocrystal. In that regard, as shown in FIG. 1A, the functionalized nanocrystals comprising a coat of diaminocarboxylic acid ("FN") unexpectedly show a significant increase in stability in an aqueous environment compared to quantum dots having an outer layer of just the

capping compound ("W-SN), when exposed over time to identical conditions of an oxidizing environment (e.g., light and air). Additionally, as shown in FIG. 1B, functionalized nanocrystals containing a coat of diaminocarboxylic acid ("FN") unexpectedly result in a significant decrease in non-specific binding compared to quantum dots having an outer layer of just the capping compound ("W-SN), when each were contacted with a surface that is both hydrophilic and hydrophobic (e.g., as may be encountered in a detection system), followed by washing of the surface, followed by detection of residual nanocrystals (as measured by number of events of fluorescence versus the intensity of fluorescence; using a fluorescence microscope with a video camera attachment, time of exposure- $1/30^{th}$ of a second).

Thus, in a preferred embodiment, the diaminocarboxylic acid (a) enhances the water-solubility of the functionalized nanocrystal; (b) has at least two free functional groups which are carboxyl-reactive, thereby enabling the diaminocarboxylic acid molecule to operably link to and crosslink carboxyl groups extending from the capping compound on the capped quantum dots; and (c) once operably linked to the capping compound, has one or more free functional groups which can be used for operably linking affinity ligand thereto. Additionally, a free carboxylic acid group on the diaminocarboxylic acid will remain as a site for attachment (operably linking) of other molecules to the diaminocarboxylic acid layer. In a more preferred embodiment, the diaminocarboxylic acid comprises lysine (2,6-diaminohexanoic acid).

For operably linking diaminocarboxylic acid to the capping compound of capped quantum dots, commercially avail-

able crosslinking agents and methods known to those skilled in the art may be used. For example, and as illustrated in FIG. 2, mercaptoacetic acid-capped nanocrystals were dissolved in an aqueous buffer system (pH of about 7). The buffer may comprise such buffers as PBS or HEPES; however, the presence of phosphate may dramatically decrease the lifetime of the crosslinking agent. To the capped quantum dots was added EDC (1-ethyl-3-[3-dimethylaminopropyl] carbdiimide) and sulfoNHS (sulfo-N-hydroxysuccinimide) in 500-1000 times excess. The resulting solution was stirred at room temperature for 30 minutes. Mercaptoethanol was added to neutralize unreacted EDC at 20 mM concentration and stirred for 15 minutes. The entire solution was then added dropwise, with stirring, to a solution of lysine (large excess) in the same buffer; and the mixture was stirred for 2 hours at room temperature. Ethanolamine (30 mM) was added to quench the reaction; and the mixture was stirred for 30 minutes at room temperature or left overnight at 4°C. The solution was centrifuged to remove any precipitated solids, and then ultrafiltered through a 30kD MW centrifugal filter. The resultant concentrated, functionalized nanocrystals can be solubilized in an aqueous solution of choice. Once solubilized, the resulting solution can be stored in an amber vial under an inert gas to prevent flocculation.

In another embodiment, as also illustrated in FIG. 2, the functionalized nanocrystals comprised of a first layer comprising capping compound and a second layer comprising diaminocarboxylic acid, is further functionalized by the addition of affinity ligand. As an illustrative example, a protein (glycoprotein, peptide, lipoprotein, etc.) having a free carboxyl-reactive group (e.g., an amine group)

can be operably linked to the free carboxyl group of the diaminocarboxylic acid of the functionalized nanocrystals using methods known in the art. For example, an affinity ligand selected from the group consisting of avidin, a monoclonal antibody, an F'ab fragment, or a lectin (e.g., wheat germ agglutinin) may be operably linked using EDC and sulfo-NHS using the general methods as previously described herein. More particularly, EDC functions to activate at least one reactive functionality (e.g., a carboxylate) to catalyze its reaction with another reactive functionality such as the amine group of a protein. The functionalized nanocrystals (1 ml, 8.1×10^{-9} mol) were esterified by treatment with EDC (8.1×10^{-6} mol), followed by treatment with sulfo-NHS (8.9×10^{-6} mol) at ambient temperature in buffered aqueous solution (at about pH 7.4) for 30 minutes. 2-mercaptoethanol was added to the solution at a concentration of 20 mM, and the mixture was stirred for 15 minutes to quench any unreacted EDC. Using a lectin wheat germ agglutinin (WGA) as an exemplary affinity ligand, the nanocrystals were then contacted with WGA (8.1×10^{-9} mol in PBS, 1 mg/ml) with vigorous stirring, and the reaction mixture was stirred for 2 hours (e.g., conditions sufficient to form an amide bond between the EDC-activated carboxylates of the diaminocarboxylate layer and the amine groups on WGA in forming functionalized nanocrystals which are water-soluble and have lectin operably linked thereto). Ethanolamine was added at a concentration of 30 mM to quench the coupling reaction, and the reaction mixture was stirred for 30 minutes. The resulting solution was then filtered through a 30 kD molecular weight cutoff centrifugal filter to remove excess reagents. The concentrated material was then diluted

to 1 ml in buffer (e.g., PBS) or other suitable aqueous solution. Essentially, the same procedure can be used to operably link avidin, an antibody, or other affinity ligand having at least one free carboxyl-reactive group.

In illustrating an embodiment of a method of using the functionalized nanocrystals, it may be desirable to attach one or a plurality of oligonucleotides to the functionalized nanocrystals for subsequent use in a nucleic acid probe hybridization detection system. In one illustration of this embodiment, the functionalized nanocrystals comprise avidinylated, functionalized nanocrystals (e.g., (CdX) core/YZ shell, capped with the capping compound, coated with diaminocarboxylic acid that is operably linked to the capping compound, followed by addition of avidin which is operably linked to the diaminocarboxylic acid) which are then contacted with, and operably linked to, a plurality of molecules of the desired oligonucleotide, each of which contains one or more biotin molecules (including native biotin or a biotin derivative having avidin-binding activity; e.g., biotin dimers, biotin multimers, carbo-biotin, and the like). Preferably, the oligonucleotides are biotinylated at a single terminus of the strand. Using methods known to those skilled in the art, biotin molecules can be added to or incorporated in a nucleotide strand, and even localized to one terminus, such as by directing synthesis of the nucleotide strands with nucleotides and biotin-nucleotides, or by biotinylating the 5' aminogroup of the nucleotide with sulfo-NHS-biotin. Thus, by contacting avidinylated, functionalized nanocrystals with biotinylated oligonucleotides, formed is a functionalized nanocrystal having a plurality of oligonucleotides extending therefrom (e.g., through the

biotin-avidin binding, the plurality of oligonucleotides become operably linked to the functionalized nanocrystals). These functionalized nanocrystals may then be used as probes in a nucleic acid probe hybridization detection system using standard methods known to those skilled in the art.

EXAMPLE 2

In another embodiment of the functionalized nanocrystals according to the present invention, the functionalized nanocrystals comprise quantum dots with a first layer comprising the capping compound, a second layer comprising diaminocarboxylic acid, and a third layer comprising an amino acid. Functionalized nanocrystals comprising capping compound, and diaminocarboxylic acid may be produced using the methods outlined in Example 1, and FIG. 2 herein. These functionalized nanocrystals are further functionalized by the addition of another layer comprising an amino acid, such as illustrated in FIG. 3. FIG. 3 illustrates the addition of an additional layer of an amino acid wherein the amino acid comprises a diaminocarboxylic acid. In this illustration, the diaminocarboxylic acid molecules of the third layer can operably link, and crosslink, the free carboxyl groups of the diaminocarboxylic acid molecules of the second layer. However, it is noted that with each diaminocarboxylic acid layer added, the number of free functional groups for reaction to operably link with a subsequent carboxylic acid layer or affinity ligand is reduced. If, for example, an affinity ligand is to be operably coupled to diaminocarboxylic acid comprising a third layer, a reduction in the number of free functional groups for reaction with the affinity ligand may be desira-

ble, particularly if it is desired to operably link relatively fewer molecules of the affinity ligand to the functionalized nanocrystals (e.g., because of one or more of the size, chemical characteristics, and specificity of the affinity ligand, or substrate to which the affinity lignd binds). However, if a maximum number of affinity ligands is desired to be operably linked to the functionalized nanocrystals, it may be disadvantageous to use a third layer comprising an amino acid comprising a diaminocarboxylic acid. If a maximum number of affinity ligands is desirable, alternative embodiments include: (a) operably linking the affinity ligand to functionalized nanocrystals comprising quantum dots, the capping compound, and the diaminocarboxylic acid; or (b) operably linking a third layer (comprising an amino acid comprising monoaminocarboxylic acid operably linked to the diaminocarboxylic acid), and then operably link the affinity ligand to the functionalized nanocrystals via the free carboxyl group of the monoaminocarboxylic acid. Thus, various factors, such as the nature of the affinity ligand to be operably linked, may guide the choice of a carboxylic acid for a third layer in further functionalizing the nanocrystals according to the present invention.

As illustrated in FIG. 3, functionalized nanocrystals comprising quantum dots, capping compound, diaminocarboxylic acid, are mixed with EDC and sulfo-NHS in 500-1000 times excess. The resulting solution is stirred at room temperature for 30 minutes. Mercaptoethanol is added to neutralize unreacted EDC at 20 mM concentration and stirred for 15 minutes. The entire solution is then added dropwise, with stirring, to a solution of an amino acid compris-

ing a diaminocarboxylic acid (e.g., lysine in large excess) in the same buffer; and the mixture is stirred for 2 hours at room temperature. Ethanolamine (30 mM) is added to quench the reaction; and the mixture is stirred for 30 minutes at room temperature or left overnight at 4°C. The solution is centrifuged to remove and precipitate solids, and then ultrafiltered through a 30kD MW centrifugal filter. The resultant concentrated, functionalized nanocrystals can be solubilized in an aqueous solution of choice. This process can also be used to add a third layer comprising an amino acid comprising a monoaminocarboxylic acid rather than a diaminocarboxylic acid. In either case, functionalized nanocrystals comprising a third layer comprising an amino acid may be further functionalized by operably linking affinity ligand to the free amine reactive group(s) (or other free reactive groups) of the amino acid comprising the third layer using methods previously described herein. Using similar methods as those outlined above, diaminocarboxylic acid may be operably linked to a capping compound comprising mercapto-functionalized amine, and more particularly, by the use of a linker.

EXAMPLE 3

In a method of detection of a target substrate using the functionalized nanocrystals according to the present invention, the functionalized nanocrystals are placed in contact with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand of the functionalized nanocrystals has binding specificity. Contact, and subsequent binding, between the affinity ligand of the functionalized nanocrystal and the sub-

strate, if present in the sample, in a detection system results in complexes comprising the functionalized nanocrystal-substrate which can emit a detectable signal for quantitation, visualization, or other form of detection. Upon formation of the complexes comprising the functionalized nanocrystal-substrate, the detectable signal emitted therefrom may be detected by first exposing the complexes formed in the detection system to a wavelength spectrum of light (visible, or UV, or a combination thereof) that is suitable for exciting the functionalized nanocrystals to emit a fluorescence peak. The peak is then detected, or detected and quantitated, by appropriate detection means (e.g., photodetector, filters, fluorescence microscope, and the like). Quantitation of the amount of substrate present is directly related to the intensity of the emitted fluorescence peak. As known to those skilled in the art of nanocrystals, the absorbance peak and fluorescence peak emissions depend on such factors which include, but are not limited to, the chemical nature, and size, of the functionalized nanocrystals. For example, functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 68.4 angstroms (A) may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color orange) at 609nm which may be detected using appropriate detection means. Functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 53.2 A may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color yellow) at 545 nm which may be detected using appropriate

detection means. Functionalized CdSe/ZnS nanocrystals having a substantially uniform core size comprising a diameter of about 46.6 A may be excited with light in the spectral range of from about 400nm to 500nm, and emit a fluorescence peak (corresponding to the color green) at 522 nm which may be detected using appropriate detection means. Detection may be by detection means comprising a scanner or reader or other analytical instrument which can detect fluorescence peaks in the range of about 410 nm to about 750 nm; and, optionally (when more than one color is used in the detection system), distinguish between discrete fluorescence peaks within that range. In the class of nanocrystals used in the present invention, many sizes of which can be excited with a single excitation light source, resulting in many emissions of colors that can be detected simultaneously and distinctly. Thus, for example, it will be apparent to those skilled in the art that more than one target substrate may be detected in a detection system simultaneously by using more than one uniform size of functionalized nanocrystals; with each uniform size having an affinity ligand operably linked thereto which has a different binding specificity (hence can detect a different target substrate) than the affinity ligand operably linked to functionalized nanocrystals of a different uniform size. As will be apparent to one skilled in the art, the detection system may include, but is not limited to, one or more of an affinity assay (e.g, immunoassay such as an ELISA), fluorescent staining (e.g., immunofluorescence staining on a glass slide), flow cytometry, nucleic acid hybridization assay, molecular sorting (e.g., cell sorting by flow cytometry), and the like.

In one illustration of this embodiment, functionalized nanocrystals, comprising diaminocarboxylic acid which is operably linked to the capping compound, are further by the addition of affinity ligand, comprising lectin WGA (wheat germ agglutinin) which is operably linked to the diaminocarboxylic acid, by using the methods outlined herein in Example 1 ("WGA-labeled, functionalized nanocrystals). To a tube containing approximately 70,000 cells of Met-129 cancer cell line (chemically induced murine mammary carcinoma) was added 200 μl of the WGA-labeled, functionalized nanocrystals, and the mixture was then rotated gently on a platform mixer. Met-129 cells have one or more cell surface glycoproteins with either terminal N-acetylglucosamine residues or with terminal sialic acid residues (e.g., mucin) which may be reactive with WGA. After 10 minutes, a drop of the mixture was placed on a microscope slide, and covered with a coverslip. Examination of the sample with a fluorescence microscope revealed that the Met-129 cells aggregated together, with the outlines of the cells clearly visible by fluorescent staining with the WGA-labeled, functionalized nanocrystals. There was very little background fluorescence remaining in the reaction media. After 30 minutes, another sample was examined, and again at 2 hours. Both of the latter samples showed agglutination of the cells, with fluorescent staining of the outside cell walls by the WGA-labeled, functionalized nanocrystals.

As a negative control for the staining mediated by the WGA-labeled, functionalized nanocrystals, unlabeled functionalized nanocrystals were added to a tube containing Met-129 cells. At 10 minutes and 30 minutes, a very low level of non-specific staining of cells was observed. In a

positive control reaction, WGA-labeled with Oregon Green fluorescent dye was added to a tube containing Met-129 cells. At each sample time, the cells were observed as large, brightly stained aggregates. However, the cell media retained a high level of background fluorescence.

The foregoing description of the specific embodiments of the present invention have been described in detail for purposes of illustration. In view of the descriptions and illustrations, others skilled in the art can, by applying, current knowledge, readily modify and/or adapt the present invention for various applications without departing from the basic concept, and therefore such modifications and/or adaptations are intended to be within the meaning and scope of the appended claims.

What is claimed:

1. A water-soluble, functionalized nanocrystal comprising: a quantum dot having a core and a shell; a capping compound operably linked to the quantum dot; and a diaminocarboxylic acid which is operably linked to the capping compound.

2. The water-soluble, functionalized nanocrystal according to claim 1, wherein the core comprises CdSe.

3. The water-soluble, functionalized nanocrystal according to claim 1, wherein the shell comprises ZnS.

4. The water-soluble, functionalized nanocrystal according to claim 1, wherein the capping compound comprises mercaptocarboxylic acid.

5. The water-soluble, functionalized nanocrystal according to claim 1, wherein the diaminocarboxylic acid forms a coating over the capping compound.

6. The water-soluble, functionalized nanocrystal according to claim 1, wherein and the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

7. The water-soluble, functionalized nanocrystal according to claim 1, further comprising affinity ligand which is operably linked to the diaminocarboxylic acid.

8. The water-soluble, functionalized nanocrystal according to claim 7, wherein the affinity ligand forms a layer over the diaminocarboxylic acid.

9. The water-soluble, functionalized nanocrystal according to claim 7, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

10. A water-soluble, functionalized nanocrystal comprising: a quantum dot; a capping compound operably linked to the quantum dot; diaminocarboxylic acid which is operably linked to the capping compound; and amino acid which is operably linked to the diaminocarboxylic acid.

11. The water-soluble, functionalized nanocrystal according to claim 10, wherein the core comprises CdSe.

12. The water-soluble, functionalized nanocrystal according to claim 10, wherein the shell comprises ZnS.

13. The water-soluble, functionalized nanocrystal according to claim 10, wherein the capping compound comprises mercaptocarboxylic acid.

14. The water-soluble, functionalized nanocrystal according to claim 10, wherein the amino acid forms a coating over the diaminocarboxylic acid.

15. The water-soluble, functionalized nanocrystal according

to claim 10, wherein the amino acid comprises a diaminocarboxylic acid.

16. The water-soluble, functionalized nanocrystal according to claim 15, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

17. The water-soluble, functionalized nanocrystal according to claim 10, wherein the amino acid comprises a monoaminocarboxylic acid, and the monoaminocarboxylic acid is selected from the group consisting of glycine, serine, threonine, cysteine, β-alanine, homoserine, γ-aminobutyric acid, and homocysteine.

18. The water-soluble, functionalized nanocrystal according to claim 10, further comprising affinity ligand which is operably linked to the amino acid.

19. The water-soluble, functionalized nanocrystal according to claim 18, wherein the affinity ligand forms a layer over the amino acid.

20. The water-soluble, functionalized nanocrystal according to claim 18, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

21. A water-soluble, functionalized nanocrystal comprising: a quantum dot; a capping compound operably linked to the quantum dot, wherein the capping compound comprises a mercapto-functionalized amine; and diaminocarboxylic acid which is operably linked to the capping compound.

22. The water-soluble, functionalized nanocrystal according to claim 21, wherein a linker is used to operably link the diaminocarboxylic acid to the capping compound.

23. The water-soluble, functionalized nanocrystal according to claim 21, wherein the diaminocarboxylic acid forms a coating over the capping compound.

24. The water-soluble, functionalized nanocrystal according to claim 21, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

25. The water-soluble, functionalized nanocrystal according to claim 21, further comprising affinity ligand which is operably linked to the diaminocarboxylic acid.

26. The water-soluble, functionalized nanocrystal according to claim 25, wherein the affinity ligand forms a layer over the diaminocarboxylic acid.

27. The water-soluble, functionalized nanocrystal according to claim 25, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

28. The water-soluble, functionalized nanocrystal according to claim 21, further comprising an amino acid which is operably linked to the diaminocarboxylic acid.

29. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid forms a layer over the diaminocarboxylic acid.

30. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid comprises a diaminocarboxylic acid.

31. The water-soluble, functionalized nanocrystal according to claim 30, wherein the diaminocarboxylic acid is selected from the group consisting of lysine, asparagine, glutamine, arginine, citrulline, ornithine, 5-hydroxylysine, djenkolic acid, β-cyanoalanine, 3,4-diaminobenzoic acid, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, 2,5-diaminopentanoic acid, and 2,6-diaminopimelic acid.

32. The water-soluble, functionalized nanocrystal according to claim 28, wherein the amino acid comprises a monoaminocarboxylic acid, and the monoaminocarboxylic acid is selected from the group consisting of glycine, serine,

threonine, cysteine, β-alanine, homoserine, γ-aminobutyric acid, and homocysteine.

33. The water-soluble, functionalized nanocrystal according to claim 28, further comprising affinity ligand which is operably linked to the amino acid.

34. The water-soluble, functionalized nanocrystal according to claim 33, wherein the affinity ligand forms a layer over the amino acid.

35. The water-soluble, functionalized nanocrystal according to claim 33, wherein the affinity ligand is selected from the group consisting of a lectin, a monoclonal antibody, a peptide, an aptamer, a nucleic acid molecule, avidin, streptavidin, and an avidin derivative.

36. A method of using the water-soluble, functionalized nanocrystal according to claim 7 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

37. The method according to claim 36, wherein the presence of the substrate is detected, and further comprises quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

38. The method according to claim 36, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

39. A method of using the water-soluble, functionalized nanocrystal according to claim 18 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

40. The method according to claim 39, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

41. The method according to claim 39, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

42. A method of using the water-soluble, functionalized nanocrystal according to claim 25 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

43. The method according to claim 42, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

44. The method according to claim 42, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

45. A method of using the water-soluble, functionalized nanocrystal according to claim 33 in a detection system, the method comprising the steps of:

(a) contacting the functionalized nanocrystals with a sample being analyzed for the presence or absence of a substrate for which the affinity ligand has binding specificity, wherein if the substrate is present in the sample, formed are complexes comprising the functionalized nanocrystals bound to the substrate;

(b) exposing the complexes, if formed, in the detection system to an excitation light source suitable for exciting the functionalized nanocrystals of the complexes to emit a fluorescence peak; and

(c) detecting the fluorescence peak emitted by the complexes, if present, by a detection means for detecting the fluorescence peak;

wherein the detection of a fluorescence peak is indicative of the presence of the substrate.

46. The method according to claim 45, wherein the presence of the substrate is detected, further comprising quantitating the amount of substrate present by measuring the intensity of the fluorescence peak emitted.

47. The method according to claim 45, wherein the detection system is selected from the group consisting of an affinity assay, fluorescent staining, flow cytometry, nucleic acid hybridization assay, and molecular sorting.

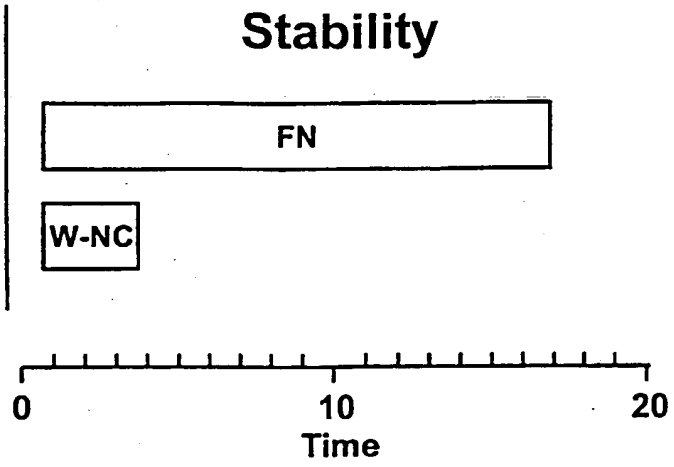
Stability
FN
W-NC
0
10
20
Time

FIG. 1A

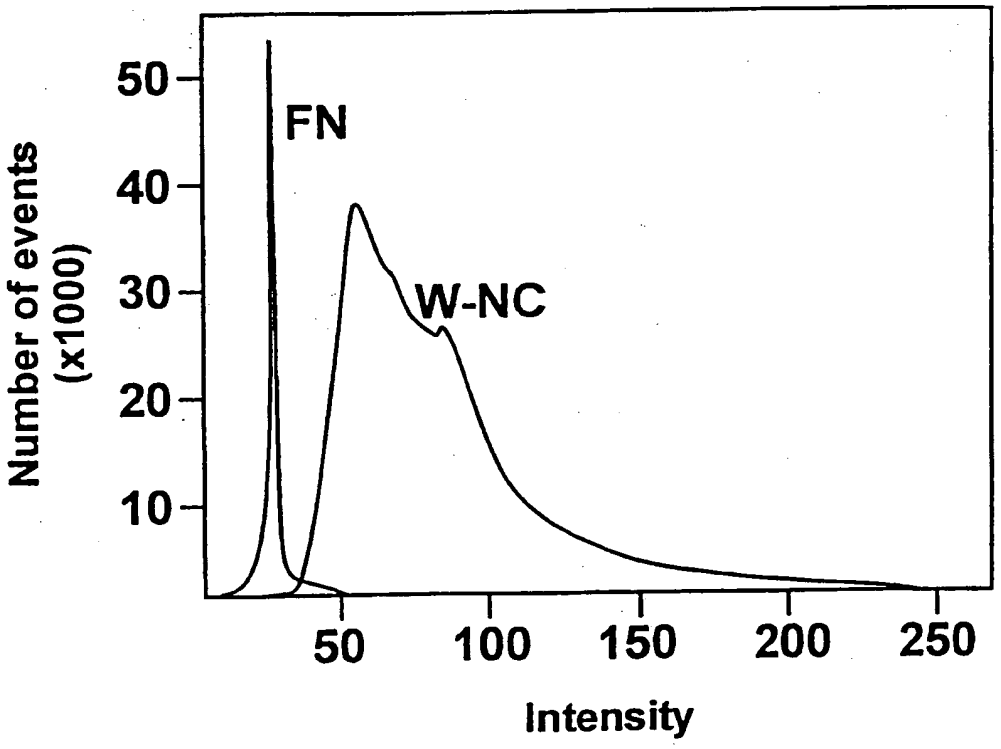
Number of events (x1000)
50
40
30
20
10
FN
W-NC
50
100
150
200
250
Intensity

FIG. 1B

2/3

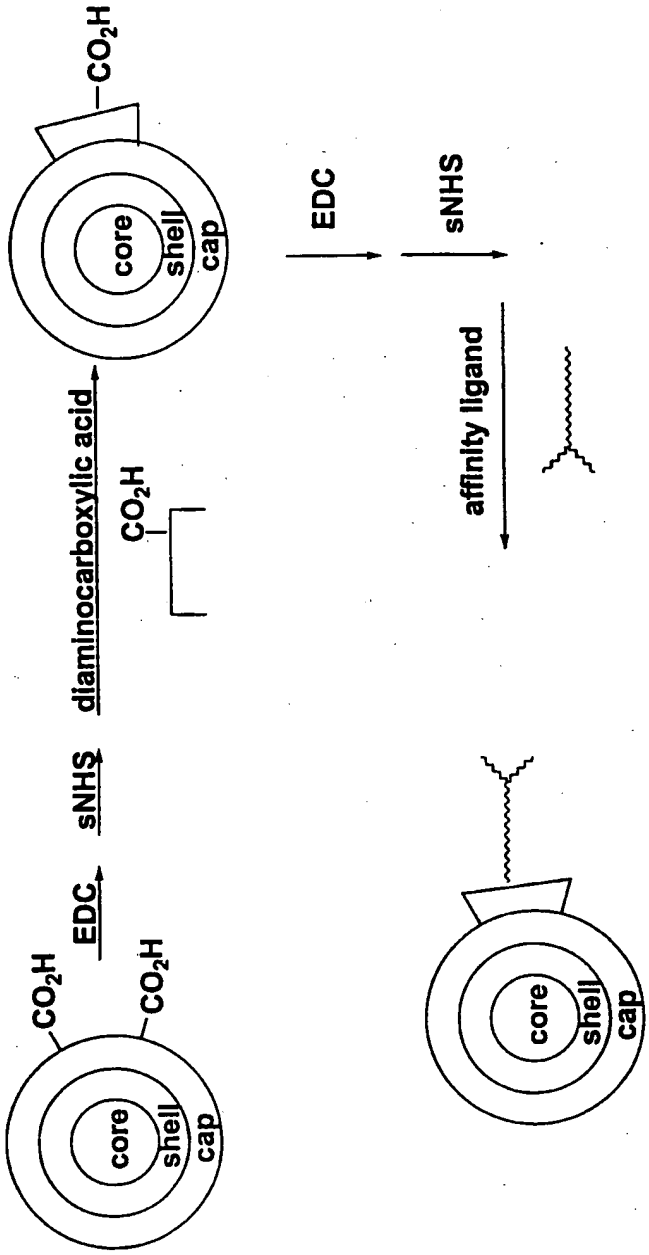
CO_2H
core
shell
cap
EDC
sNHS
affinity ligand
diaminocarboxylic acid
CO_2H
sNHS
EDC
CO_2H
CO_2H
core
shell
cap
core
shell
cap

FIG. 2

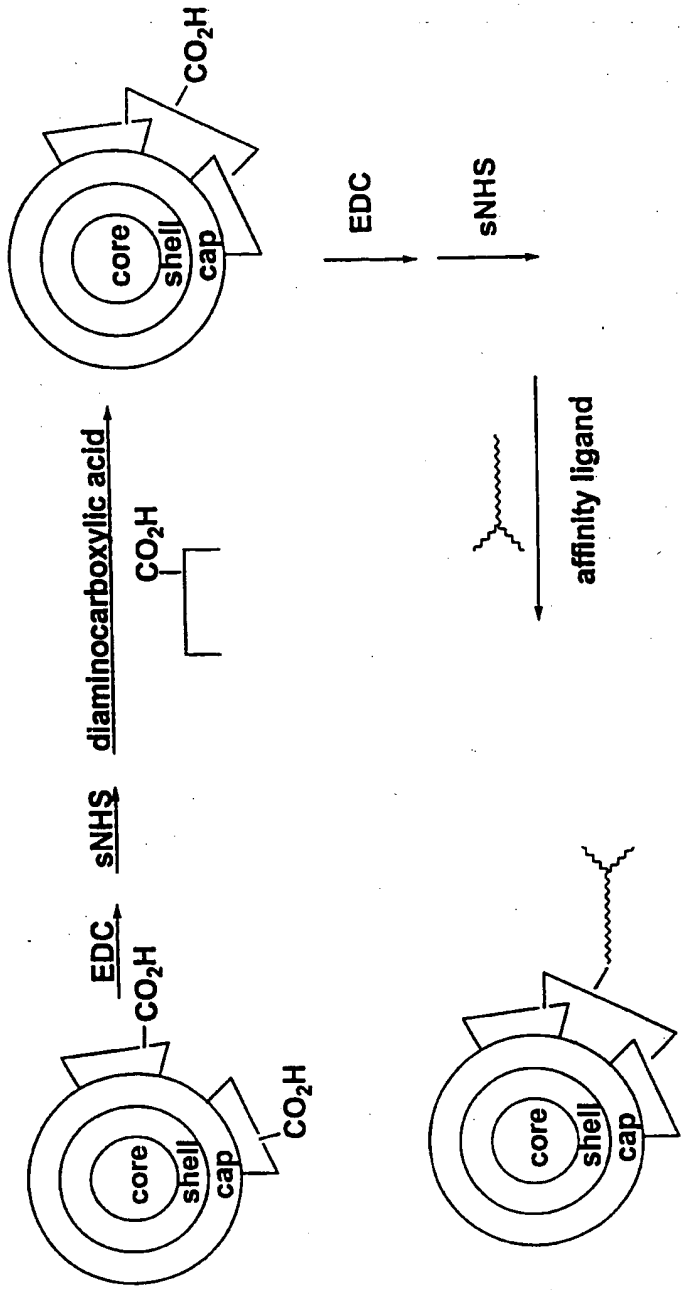
core
shell
cap
CO_2H
EDC
sNHS
diaminocarboxylic acid
affinity ligand

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26487

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/307, 361C 361R, 432R, 459.1; 356/317; 378/45, 48; 424/490; 436/546; 428/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST 1.2, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,525,377 A (GALLAGHER et al) 11 June 1996, entire document.	1-47
A	US 5,882,779 A (LAWANDY) 16 March 1999, entire document.	1-47
A	US 5,908,608 A (LAWANDY et al) 01 June 1999, entire document.	1-47
A	US 5,990,479 A (WEISS et al) 23 November 1999, entire document.	1-47
A	JP 11087689 A (EGAWA) 30 March 1999, entire document.	1-47
A	JP 11154771 A (FUJII) 08 June 1999, entire document.	1-47

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 MARCH 2000	Date of mailing of the international search report 04 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer H. THI LE Telephone No. (703) 308-2415

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26487

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 9/16; B32B 5/16; F21V 9/16; G01J 3/30; G01N 21/64, 23/02, 23/223, 33/533

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

250/307, 361C 361R, 432R, 459.1; 356/317; 378/45, 48; 424/490; 436/546; 428/403

CORRECTED VERSION*

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(54) Title: FUNCTIONALIZED NANOCRYSTALS AND THEIR USE IN DETECTION SYSTEMS

(57) Abstract

Provided are compositions comprising water–soluble, functionalized nanocrystals. The water–soluble functionalized nanocrystals comprise quantum dots capped with a layer of a capping compound, and further comprise, by operably linking and in a successive manner, one or more additional compounds. Preferably, an additional compound comprises diaminocarboxylic acid which is operatively linked to the capping compound, and may further comprise an amino acid, and affinity ligand, or a combination thereof. Also provided are methods of using the functionalized nanocrystals having an affinity ligand to detect the presence or absence of a target substrate in a sample by contacting the functionalized nanocrystals with the sample so that complexes are formed between the functionalized nanocrystals and substrate, if the substrate is present; exposing the complexes in the detection system to an excitation light source, and detecting the emitted fluorescence peak.

*(Referred to in PCT Gazette No. 37/2000, Section II)

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